

Remarks

The Applicants address the Examiner's remarks in the order she presented them. Claims 1-23 are pending. The Applicants have canceled claims 6, 15, and 22, and added claims 24 and 25. The Examiner withdrew claims 7 and 8 as part of a restriction requirement.

Priority

Applicants submit herewith an English translation of U.S. Provisional Application No. **60/270,593** filed on **February 23, 2001**. Also submitted herewith is a statement by the translator of the accuracy of the translation.

The Title

The Examiner objected to the title of the invention, stating that the title is not descriptive. The Applicants disagree, but they have nonetheless amended the title of the invention. They respectfully submit that the amended title clearly describes the claimed invention.

Use of Trademarks

The Applicants note the Examiner's admonition regarding their use of trademarks. They point out that they recite trademarks in connection with the "®" symbol, alerting the reader that the mark to which the symbol is appended is a federally registered trademark. If the Examiner finds an exception to the Applicants' practice, they invite her to identify it, whereupon they will make the necessary correction.

The Provisional Double-Patenting Rejection

The Examiner rejected claims 1-6 and 9-23, stating that they conflict with the claims of Application Nos. 10/076,631 and 10/076,632. The Applicants disagree that there is any conflict, but for the present merely note that the rejection is a provisional one, as no patents have issued from any of the applications in question.

The Rejection Under § 112, First Paragraph

The Examiner rejected claims 1-6 and 9-23 under 35 U.S.C. § 112, first paragraph, stating that they failed to comply with the written description. Specifically, the Examiner, while admitting that the “specification describes several alternative members for ‘S’”, stated that it “fails to describe alternatives for the structures of ‘Asm’, ‘Rn’, ‘Y’, ‘P’ and ‘T’”. Office Action, June 16, 2004, at pages 7-8.

The Applicants respectfully disagree, but, even if the Examiner’s description is correct, it is of no consequence: it does not matter what As_m, P, or Y are. As_m represents a nucleic acid sequence comprising a codon, P represents a promoter, and Y represents a protein of interest. They may be *any* nucleic acid sequence, *any* sequence encoding a promoter, and *any* sequence encoding a protein of interest (all within a reasonable scope that one of skill in the art would recognize, of course) because the invention, as the specification makes clear, does not lie within those sequences. One need not describe in detail such components of an invention in order to be considered in possession of it. As to R_n and T, the Applicants note that they are no longer an issue in view of the amendments to claims 1 and 2.

That leaves F. The claims, as amended, define F as a hirudin derivative (claim 1), such as lepirudin, Ser-hirudin or Ala-hirudin (claim 4). The Examiner alleges that “the specification utterly fails to teach a single protein ‘F’ that ‘allows secretion of a protein by Y into a fermentation medium,’” Office Action, at 8, but it is unclear what the Examiner means by this objection. If the Examiner is alleging that the specification fails to recite examples of “F,” we need point only to the Examiner’s own admission that the specification teaches as examples of F “specific hirudin derivatives.” Office Action, at 8. Indeed, the application expressly recites such derivatives in the claims. If the Examiner’s position is that the hirudin derivatives the Applicants identify do not do what the Applicants say they do – that is, do not allow secretion of a protein into a fermentation medium – the Applicant’s respectfully draw her attention to the Examples, and in particular Example 1, which show the contrary.

In view of the foregoing, the Applicants respectfully request that the Examiner withdraw the rejection of claims 1-6 and 9-23 under § 112, first paragraph.

The Second Rejection Under § 112, First Paragraph

The Examiner rejected claims 1-6 and 9-23 under 35 U.S.C. § 112, first paragraph, stating that the specification does not enable a person skilled in the art to

make and use the invention commensurate in scope with the claims. The Examiner observes that the specification is

enabling for the particularly disclosed nucleic acids encoding fusion proteins, plasmids, bacterial host cells and methods of fermentative production in a bacterial cell and isolation of the fusion proteins thereof from a culture supernatant as particular set forth in the Examples of 1-9 of the specification, and the Applicants agree. They submit that their amendments to the claims satisfactorily address the grounds of the Examiner's rejection, and therefore respectfully request that she withdraw it.

The Rejection Under § 112, Second Paragraph

The Examiner rejected claims 1-6 and 9-23 under § 112, second paragraph, stating that the claims are indefinite.

As to claim 1, the Applicants have amended the limitation relating to "As_m" and believe that the amendment satisfactorily addresses the Examiner's objections to that limitation.

As to claim 3, the Examiner objected that the phrases "smompa derived from," "ecoompc derived from," "af009352 derived from," "aeoynxa derived from," are "not described nor specifically defined in the specification." The Applicants respectfully disagree. The sequence represented by "smompa" is defined at ¶ 128 as a "[s]ignal sequence smompa derived from the ompA gene for major outer membrane protein of Serratia marcescens (GenEMBL data base locus: SMOMPA, 1364 bp DNA BCT Mar. 30, 1995)." The sequence represented by "ecoompc" is similarly defined at ¶ 129; "af009352" at ¶ 130; and "aeoynxa" at ¶ 131. These definitions enable anyone of ordinary skill in the art to make and use the sequences in a manner fully commensurate with the scope of the claims.

As to claim 4, the Examiner objected that "Ser-hirudin" and "Ala-hirudin" "have no meaningful interpretation." But these derivatives are known and readily available from commercial suppliers, as the specification points out. European Patent Application No. 86117098.3 (the Applicants enclose a copy) describes the naming convention of hirudin variants this way: "The variants differ in structure from each other by a number of amino acids (especially, the N-terminal sequence of HV1 s Val-Val-Tyr, that of HV2 is Ile-Thr-Thr and that of 'des-(Val)₂-hirudin' is Thr-Tyr)," at page 1, paragraph 2. Hence, "Ser-hirudin" and "Ala-hirudin" are hirudins with an N-

terminal "Ser" and "Ala," respectively, instead of the "Val-Val" and "Ile-Thr" N-terminus of the hirudin variants exemplified in the European application.

Claim 6 is no longer at issue because the Applicants have canceled it.

The Applicants' amendments to claims 14, 16-21, and 23 address the Examiner's objections to those claims. The Applicants have canceled claims 15 and 22.

As to claims 2-5, the Examiner objected that the term "signal sequence" cannot be "used by the claim to mean 'a nucleic acid sequence that increases yield.'" The Applicants do not use the term in the manner the Examiner suggests. The limitation "a nucleic acid sequence that increases yield" is a *property* of the signal sequence, not the definition of it. The "signal sequence" is, in fact, a signal sequence, as the art understands that term to be. It merely has the additional property recited by the claim. The Applicants respectfully submit that the Applicants' use of the term fully comports with its ordinary meaning, and, hence, the requirements of § 112.

In view of the foregoing, the Applicants respectfully request that the Examiner withdraw the rejection under § 112, second paragraph as to claims 1-6 and 9-23.

The Examiner rejected claims 1, 2, 6, 9-15, 17, 22, and 23 under 35 U.S.C. § 102 (b), stating that WO 91/09125 (Dawson *et al.*) anticipates the claims. The Applicants respectfully submit that their amendments to the claims obviate the rejection. The Dawson reference does not disclose or suggest the use of hirudin derivatives in the fusion proteins it discloses. The Applicants therefore respectfully request the Examiner withdraw the rejection under § 102(b).

The Applicants respectfully submit that the claims, as amended, are in condition for allowance, and respectfully request early, favorable action on the application. Should the Examiner believe that an interview would advance the prosecution of this application, the Applicants invite her to contact the undersigned at 908.231.3444.

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Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Joel B. German', is written over a horizontal line.

Joel B. German, Reg. No., 48,676
Attorney for Applicants

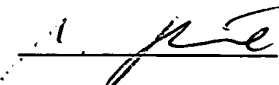
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UNITED STATES PATENT AND TRADEMARK OFFICE

I, Martin Hermann BRUNE BSc, MSc, PhD,
translator to RWS Group Ltd, of Europa House, Marsham Way, Gerrards Cross,
Buckinghamshire, England declare;

1. That I am a resident of the United Kingdom of Great Britain and Northern Ireland.
2. That I am well acquainted with the German and English languages.
3. That the attached is, to the best of my knowledge and belief, a true translation into the English language of the specification in German filed with the application for a patent in the U.S.A. on 23 February 2001
under the number 270593/60
4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.


For and on behalf of RWS Group Ltd
The 24th day of August 2004

Fusion protein for the secretion of a protein of interest into bacterial supernatants

5 Description

The invention relates to fusion proteins comprising a fusion part and a protein of interest, the combination of the two proteins leading to the fusion protein being secreted into the supernatant of a bacterial host and the protein of interest being present in its correct three-dimensional structure. The gene sequence for the fusion protein is part of an expression cassette which allows expression in a bacterial host. The invention relates to a process for the fermentation, expression and work-up of such a fusion protein using the expression cassette, to a plasmid containing the expression cassette, to a bacterial host cell containing the expression cassette integrated into the chromosome and/or as a replicon, for example as a plasmid, to said fusion protein with hirudin or a derivative thereof as the fusion part, to a process for producing insulin or an insulin derivative and to the use of the expression cassette in the processes for preparing a fusion protein from hirudin or derivatives thereof and for producing insulin or an insulin derivative.

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The development of optimized processes for producing pharmaceuticals on the basis of recombinant proteins represents a task which has to do justice to two points of view, if possible. First, a process ought to be as cost-effective as possible and secondly, the product ought to be of the highest purity.

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In this connection, the choice of expression system determines the course of the particular production process, and it is obvious to the skilled worker that the development of novel protein-chemical techniques and the wide variety of biochemical possibilities and new combinations of known techniques always make improvements of existing processes possible.

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The properties of a desired protein determine in a decisive way the choice of the host cell system used for the synthesis. Bacteria such as *E. coli* represent the system with the aid of which it is possible to rapidly produce proteins with crude yields of several grams in inexpensive media. The system comes in useful especially

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for proteins which need not be modified and which can be renatured in vitro to their biologically active form. For proteins which are needed in high quantities, such as insulin for example, expression rates leading to intracellular accumulation of the protein in the form of inclusion bodies are aimed at. After cell lysis, the protein is dissolved and then, in further process steps, folded. However, the process of folding is not quantitative. Reasons for this may be irreversible damage during inclusion body formation, corresponding damage during cell lysis and errors during folding. "Wrongly" folded or modified molecules then have to be removed in further separation steps. This has an adverse effect on production costs. In addition, traces of said molecules reappear also in the final product. Since pharmaceuticals are subject to high criteria of purity, an appropriately careful and cost-intensive purification is necessary. Owing to the favorable cost / crude yield ratio, processes allowing export by *E. coli* of the protein of interest in correctly folded form into the culture medium would be of desirable. However, this has been successful only in exceptional cases up until now.

The international patent application PCT/EP00/08537 describes such an exception. Synthesis and export of lepirudin, the active ingredient of the pharmaceutical Refludan[®], by *E. coli* in gram quantities was successful when using specific signal sequences for exporting. The German patent application No. 100 33 195.2 (unpublished) describes a bifunctional protein composed of hirudin and hirudin derivatives and of factor Xa inhibitor from ticks and derivatives thereof. Said protein can likewise be synthesized and exported by *E. coli* with high yields. As an addition to this finding, it was then surprisingly found that hirudin is exported with high yields not only as a fusion protein with TAP but also as part of a fusion protein with polypeptides such as proinsulin derivatives, that it is biologically active and that surprisingly a fusion partner such as proinsulin is present in the correct three-dimensional structure. This unexpected result leads to the possibility of more cost-effective production of, for example, insulin by bacterial host/vector systems, since the step of in vitro refolding after intracellular expression, which is associated with losses in yield which are not negligible, can be dispensed with and in this way a simpler protein purification process results. Another advantage is that chaotropic aids which are added to dissolve the fusion protein in traditional processes for the

production of insulin in *E. coli* are not required. Ecologically, this leads to less environmental pollution by avoiding the corresponding waste.

Leeches of the *Hirudo* type have developed, for example, various isoforms of the thrombin inhibitor hirudin. Hirudin has been optimized for pharmaceutical requirements by artificial variation of the molecule, for example exchange of the N-terminal amino acid (e.g. EP-A 0 324 712).

The invention includes the use of hirudin and hirudin variants for the formation of fusion proteins, for example with simian proinsulin or derivatives thereof. Particular embodiments of the invention use one of the natural hirudin isoforms (the natural isoforms together are denoted "hirudin"). Natural isoforms are, for example, Val-Val-hirudin or Ile-Thr-hirudin. Other embodiments of the invention use a variant of a natural hirudin isoform. A variant is derived from a natural hirudin isoform but contains, for example, additional amino acids and/or amino acid deletions and/or amino acid exchanges compared with the natural isoform. A hirudin variant may contain alternating peptide segments of natural hirudin isoforms and new amino acids. Hirudin variants are known and are described, for example, in DE 3 430 556. Hirudin variants are commercially available in the form of proteins (Calbiochem® Biochemicals, Cat.no.377-853, -950-960).

Insulin is a polypeptide of 51 amino acids which are distributed between two amino acid chains: the A chain with 21 amino acids and the B chain with 30 amino acids. The chains are connected to one another by 2 disulfide bridges. Insulin compositions have been used for many years for the therapy of diabetes. This includes the use not only of naturally occurring insulins but also of insulin derivatives and analogs.

Insulin derivatives are derivatives of naturally occurring insulins, namely human insulin or animal insulins, which differ from the corresponding, otherwise identical naturally occurring insulin by substitution of at least one naturally occurring amino acid residue and/or addition of at least one amino acid residue and/or organic residue.

In general, insulin derivatives have a slightly modified action compared with human insulin.

Insulin derivatives having an accelerated onset of action are described in

- 5 EP 0 214 826, EP 0 375 437 and EP 0 678 522. EP 0 124 826 inter alia relates to substitutions of B27 and B28. EP 0 678 522 describes insulin derivatives which have at position B29 various amino acids, preferably proline, but not glutamic acid. EP 0 375 437 includes insulin derivatives with lysine or arginine at B28, which may additionally be modified at B3 and/or A21, where appropriate.

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EP 0 419 504 discloses insulin derivatives which are protected against chemical modification by modification of asparagine at B3 and of at least one other amino acid at positions A5, A15, A18 or A21.

- 15 WO 92/00321 describes insulin derivatives in which at least one amino acid at positions B1-B6 has been replaced by lysine or arginine. According to WO 92/00321, insulins of this kind exhibit a prolonged action.

- 20 When producing insulin and insulin derivatives by genetic engineering, an insulin precursor, "proinsulin", comprising B, C and A chains is frequently expressed. Said proinsulin can be converted into insulin or an insulin derivative by enzymatic or chemical removal of the C chain after appropriate and correct folding and formation of the disulfides bridges. Proinsulin is frequently expressed in the form of a fusion protein. The "unwanted" fusion partner likewise needs be removed chemically or
25 enzymatically.

It is obvious to the skilled worker that the choice of recombinant host/vector systems determines the methods for cultivation, propagation and fermentation of the recombinant cells. This is likewise a subject of the invention.

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The fusion protein shows surprisingly good solubility in acidic medium, and this leads to distinct advantages regarding the chemical workup of the protein. Firstly, many unwanted components of the supernatant are precipitated under said conditions and, secondly, peptidases or proteases are inactive. Thus, acidifying the fermentation

broth at the end of the operation makes it possible to directly separate unwanted supernatant proteins together with the host cells from the fusion protein and, in a further step, to concentrate said fusion protein. This is likewise a subject of the invention.

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At the end of the fermentation, the folding process may not yet be 100% complete. The addition of mercaptan or, for example, cysteine hydrochloride can complete the process. This is likewise a subject of the invention.

- 10 If the two proteins are fused via a linker of amino acids which are specifically recognized by endoproteases which efficiently cleave the fusion protein at no other position, then the protein of interest can be cleaved off directly in active form. In the case of insulin production, the linker between hirudin and proinsulin preferably contains arginine at the carboxy-terminal end. In simultaneous processing it is then
- 15 possible by conversion with trypsin to cleave off the fusion part and convert proinsulin to mono- or di-Arg-insulin. Said linker must be optimized in relation to insulin processing such that cleaving off the hirudin part is not slower than cleavages in the C peptide sequence or a derivative thereof which links the B and A chains of insulin. This is likewise a subject of the invention. An example of an expression
- 20 system which can be used is the vector pJF118, described in figure 1 of European patent 0 468 539.

Plasmids which [lacuna] DNA sequences encoding proinsulin or proinsulin derivatives are described, for example, in the patents EP-A 0 489 780 and

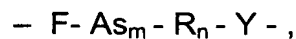
25 PCT/EP00/08537.

The plasmid pK152 which contains the sequence for hirudin according to EP-A 0 324 712 is used as source [lacuna] the DNA sequence for hirudin.

- 30 The export compatibility of the protein of interest for passing through the inner bacterial membrane of the bacterium is important for secretion. In this context, the choice of signal sequence which can be more or less optimal for different proteins is important. The patent application PCT/EP00/08537 describes a system of PCR-based signal sequence screening. This system can also be applied to fusion proteins

having hirudin as the N-terminal fusion part, since hirudin activity surprisingly remains intact and thus becomes readily detectable in the supernatant by means of the thrombin inhibition assay.

5 The invention therefore relates to a DNA encoding a fusion protein of the form



where

- 10 F is a DNA sequence coding for an amino acid sequence which allows secretion of a protein Y into a fermentation medium,
 As is a chemical bond or a DNA sequence coding for an amino acid encodable by genetic engineering,
 m is an integer from 0 - 10,
 R is a chemical bond or an arginine codon,
 15 n is 0 or 1, and
 Y is a DNA sequence coding for a protein of interest which, correctly folded, is part of the fusion protein in the fermentation medium, choosing in particular a DNA sequence coding for hirudin or a derivative thereof (F) and proinsulin or a derivative thereof (Y).

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The invention further relates to an expression cassette of the form



where

- 25 P is a promoter,
 S is a DNA sequence coding for a signal sequence allowing optimal yields,
 T is an untranslated expression-enhancing DNA sequence.

30 The invention further relates to a plasmid containing an above-described expression cassette and to a host cell containing said plasmid or to a host cell which preferably contains the expression cassette integrated into the host genome, the host cell being selected from a group comprising E.coli, B. subtilis and Streptomyces.

The invention also relates to a process for the fermentative production of a fusion protein as described above, in which process

- (a) an expressions cassette as described above is expressed in a host cell as described above and
- 5 (b) the expressed fusion protein is isolated;

in which, in particular, the supernatant is separated from the host cells to isolate the expressed protein, and the expressed protein is isolated from the supernatant; and in which a process step for concentrating the expressed protein in the supernatant after
 10 precipitation is selected from a group comprising microfiltration, hydrophobic interaction chromatography and ion exchange chromatography, and in which a particular embodiment is that isolation of the expressed protein includes a step in which components of the culture medium or the supernatant are precipitated, while the expressed protein remains in solution; and in which in a further preferred
 15 embodiment of the invention, after the fermentation, mercaptan or cysteine hydrochloride are added to the fermentation supernatant at pH 6 – 9, resulting in a free SH group concentration of from 0.05 to 2.5 mM.

A particular embodiment of the invention comprises separating the fermentation supernatant from the host cells, further culturing the host cells in fresh medium and
 20 isolating the released fusion protein from the supernatant.

The invention further relates to a process for the production of insulin or an insulin derivative, in which process

- (a) from the expressed protein which is obtained in a process as described above
- 25 (b) the protein of interest, in particular insulin or insulin derivative, is released by enzymatic or chemical cleavage and
- (c) is isolated.

The following examples which are not intended to be restrictive describe the
 30 invention in more detail.

Example 1: Construction of a lepirudin–GNSAR–simian proinsulin fusion protein, appended to the signal sequence of the oprF gene product from *Pseudomonas fluorescens*

Example 2 of the patent application PCT/EP00/08537 described an expression vector which allows expression and secretion of Refludan into the medium used for *E. coli* via the signal sequence of the *Pseudomonas fluorescens* *oprF* gene product (De, E. et al. FEMS Microbiol Lett. 127, 263–272, 1995). This vector serves to construct a Refludan–GNSAR–simian proinsulin fusion protein (GNSAR=SEQ ID NO.: 1) and is denoted pBpfu_hir.

Further starting materials are pJF118 (EP 0 468 539) and pK152 (PCT/EP00/08537) plasmid DNAs. The following oligonucleotides are required:

Primer pfuf1

5'GGTTCTCTTA TTGCCGCTAC TTCTTTCGGC GTTCTGGCAc ttacgtatac
tgactgca 3'

(SEQ ID NO.: 2)

Primer insu11hindIII

5' - TTTTTAAGCT TCATGTTTGA CAGCTTATCA T –3' (SEQ ID NO.: 3)

Primer Hir_insf1

5' ATCCCTGAGG AATACCTTCA GGGAAATTCG **GCACGATTG TG** – 3' (SEQ ID NO.: 4)

Primer Hir_insrev1

5' - **CACAAATCGT GCCGAATTC** CCTGAAGGTA TTCCTCAGGG AT –3' (SEQ ID NO.: 5)

Primer pfuf1 hybridizes with the DNA region encoding the junction of signal sequence and lepirudin in the expression vector.

The part of primer Hir_insrev1 shown in bold type hybridizes with the DNA region encoding the junction of preproinsulin and simian proinsulin sequences in plasmid pINT90d and with sequences of the 3' end of the hirudin sequence in plasmid pK152. Primer Hir_insrev1 is 100% complementary to primer Hir_insf1.

Primer Insu11HindIII marks the 3' end of the DNA region cloned in pINT90d and encoding the simian proinsulin sequence and additionally carries the hexanucleotide sequence for recognition by the restriction enzyme HindIII.

- 5 Two standard polymerase chain reactions are carried out using the Hir_insf1 / Insu11HindIII primer pair with plasmid pINT90d as template and the pfuf1/ Hir_insrev primer pair with plasmid pBpfu_hir as template. The products of both reactions are combined and an aliquot is converted in a third polymerase chain reaction with primaries pfuf1/Insu11HindIII. The result is a DNA product which contains the
- 10 sequence signal (partially)-lepirudin–GNSAR– simian proinsulin. The DNA fragment is converted using restriction enzymes BamHI and HindIII, with BamHI cleaving in the lepirudin sequence and HindIII at the 3' end of the proinsulin-encoding sequence.
- 15 In a parallel reaction, vector pBpfu is converted using the two enzymes and the large vector fragment is isolated. The isolated products of both reactions are converted in a T4 ligase reaction. Competent cells of the E. coli strain K12 Mc1061 (Sambrook et al. "Molecular Cloning" (Cold Spring Harbor Laboratory Press 1989) are transformed with the ligation mixture and plated on NA plates containing 25µg /ml ampicillin.
- 20 Plasmid DNA is isolated from transformants for characterization. At the same time, a plate with the transformants characterized in the plasmid analysis is produced for maintenance purposes. The DNA is characterized by means of restriction analysis and DNA sequence analysis. A plasmid identified as correct was denoted pBpfuHir_Ins.

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Example 2: Construction of a Ser-hirudin–GNSAR–simian proinsulin fusion protein appended to the signal sequence of S. typhimurium outer membrane protein (fimD)

- 30 The construction is carried out according to the plan described in example 1.

Example 10 of patent application PCT/EP 00/08537 describes the construction of a vector for exporting lepirudin via the signal sequence of S. typhimurium outer membrane protein (Rioux,C.R., Friedrich,M.J. and Kadner,R.J.;J. Bacteriol. 172

(11), 6217-6222 (1990)) . The resulting plasmid is denoted pBstyfim_hir for laboratory purposes. DNAs of plasmids pK152 and pINT90d serve in each case as templates.

5 The construction requires 4 primers.

The primers insu11HindIII, Hir_insf1 and Hir_insrev1 are described in example 1.

The primer styfimf1ser is newly synthesized and has the following sequence:

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5' CGGCGCTGAG TCTCGCCTTA TTTTCTCACC TATCTTTTGC **CTCT**acgtat
actgactgcaCTG 3' (SEQ ID NO.: 6)

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The DNA triplet shown in bold type indicates a serine codon. As a result, a hirudin is produced which carries serine instead of leucine at position 1 of the amino acid sequence.

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Corresponding to example 1, two standard polymerase chain reactions are carried out using the Hir_insf1 / Insu11HindIII primer pair with pINT90d DNA as template and the styfimf1ser / Hir_insrev primer pair with pK152 DNA as template. The products of both reactions are combined and an aliquot is converted in a third polymerase chain reaction with primers styfimf1ser / Insu11HindIII. The result is a DNA product which contains the sequence signal (partially)-Ser-hirudin-GNSAR-simian proinsulin. The DNA fragment is converted using the restriction enzymes

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BamHI and HindIII.

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In a parallel reaction, vector pBstyfim_Hir is converted using the two enzymes and the large vector fragment is isolated. The isolated products of both reactions are converted in a T4 -ligase reaction. Competent cells of E. coli strain K12 Mc1061 are transformed with the ligation mixture, and plasmid DNA is isolated from transformants for characterization. At the same time, a plate with the transformants characterized by plasmid analysis is produced for maintenance purposes. The DNA is characterized by means of restriction analysis and DNA sequence analysis. A plasmid identified as correct was denoted pBstyfim_SerHir_Ins.

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Example 3: Construction of an Ala-hirudin-R-simian proinsulin fusion protein appended to the signal sequence of the E. coli alkaline phosphatase precursor protein

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The E. coli alkaline phosphatase precursor has the signal sequence:

MKQSTIALAL LPLLFTPVT K A (SEQ ID NO.: 7)

10 (Shuttleworth H., Taylor J., Minton N.; Nucleic Acids Res. 14:8689, (1986)).

The peptide sequence is translated into DNA by the GCG program Backtranslate (Wisconsin Package Version 10.1, Genetics Computer Group (GCG), Madison, Wisc.) using the Coli high codon usage criteria.

15

This results in the sequence:

5'ATGAAACAGTCGACCATCGCGCTGGCGCTGCTGCCGCTGCTGTTACCCCCG
GTTACCAAAGCG 3' (SEQ ID NO.: 8)

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To clone and append this sequence to a DNA sequence coding for a hirudin which is characterized by having the amino acid alanine at position 1 (EP-A 0 448 093), said sequence is extended by the sequence shown in bold type:

25 5'**TTTTTTGAATTC**ATGAAACAGTCGACCATCGCGCTGGCGCTGCTGCCGCTGCTGTTCA
ACCCCGGTTACCAAAG -CG **GCTacgtat actgactgcaCTG** (SEQ ID NO.: 9)

Two oligonucleotide sequences which partially overlap are derived therefrom.

30 Primer phoaf1 has the sequence :

5'**CTGCTGCCGCTGCTGTTACCCCGGTTACCAAAGCG** GCTACG
TATACTGACTGCACTG -3' (SEQ ID NO.: 10)

Primer phoaf2 has the sequence:

5'TTTTTTGAATTCATGAAACAGTCGACCATCGCGCTGGCGCTGCTGCCGCTGCTG -3'
(SEQ ID NO.: 11)

The construction of the expression vector requires primers insu11HindIII , Hir_insf2
5 and Hir_insrev2 and DNAs of plasmids pK152, pINT90d and pJF118.

Primer Hir_insf2 has the sequence:

5' - ATCCCTGAGGAATACCTTCAG**cga**TTTGTGAACCAGCAC C -3'(SEQ ID NO.
12)

10

Primer Hir_insrev2 has the sequence:

5' - **GGTGCTGGTTCACAAA****tcg**CTGAAGGTA TTCCTCAGGG AT-3'(SEQ ID
NO.13)

15 Upper case letters in bold type indicate the sequence hybridizing with proinsulin,
while upper case letters in plain type describe overlap with the 3' end of the hirudin
sequence. Lower case letters underlined and in bold type represent the codon for the
linker arginine.

20 Corresponding to example 1, two standard polymerase chain reactions are carried
out using the Hir_insf1 / Insu11HindIII primer pair with pINT90d DNA as template
and the phoaf1 / Hir_insrev primer pair with pK152 DNA as template. The products
of both reactions are combined and an aliquot is converted in a third polymerase
chain reaction with primers phoa /Insu11HindIII. The result is a DNA product which
25 contains the sequence signal-Ala-hirudin-GNSAR- simian proinsulin. The DNA
fragment is converted using restriction enzymes BamHI and HindIII. In a parallel
reaction, vector pJF118 is converted using the two enzymes and the large vector
fragment is isolated. The isolated products of both reactions are converted in a T4-
ligase reaction. Competent cells of E. coli strain K12 Mc1061 are transformed with
30 the ligation mixture, and plasmid DNA is isolated from transformants for
characterization. At the same time, a plate with the transformants characterized by
plasmid analysis is produced for maintenance purposes. The DNA is characterized
by means of restriction analysis and DNA sequence analysis. A plasmid identified as
correct was denoted pNS22.

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Example 4: Thrombin inhibition assay

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The hirudin concentration is determined according to the method of Griebach et al. (Thrombosis Research 37, pp. 347 –350 , 1985). For this purpose, specific amounts of a Refludan standard are included in the measurements in order to establish a calibration curve from which the yield in mg/l can be determined directly. The biological activity is also a direct measure for correct folding of the proinsulin component of the fusion protein. Alternatively, it is possible to use a proteolytic *S. aureus* digestion and subsequent analysis in an RP-HPLC system to determine the correct S-S bridge formation.

10

15

Example 5: Expression of the fusion protein

Recombinant cells are cultivated overnight in 2YT medium (per liter: 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl) containing 100 µg/ml ampicillin. The overnight culture is diluted 1:50 with fresh medium and the cells are cultivated to a density of approximately 0.8 OD₆₀₀.

20

Expression is then induced by adding IPTG in such a way that a concentration of 0.05–2 mM is established. The cells induced in this way are incubated for a further 3-26 h.

25

After three hours, an antithrombin action of hirudin is clearly measurable in the supernatant. Said action can be attributed to secretion of the desired fusion protein, since SDS PAGE analysis, after Coomassie blue staining, reveals only in induced cells a new band which reacts in Western blot analysis with polyclonal anti-insulin antibodies. In fermentation experiments, induction is commenced only after cultivation to significantly higher optical densities. Preference is given here to synthetic media based on minimal medium.

30

Cell productivity can be increased by using the principle of bacterial milking, i.e. by carefully removing the cells, after the optimal induction time, from the supernatant and further incubating them in fresh medium to which the inducer can again be added. Insulin is then prepared in parallel from the harvested supernatant.

5

Example 6: Purification of the fusion protein

After induction has finished, the cell supernatant is adjusted to pH 2.5 – 3 and cells and supernatant components are removed by centrifugation or filtration. The supernatant of the precipitation is applied to a cation exchange column (Hyper DF) and fractionated using a linear gradient from 150 to 450 mM NaCl at pH 3.5 in the presence of 30% 2-propanol. The individual fractions are analyzed by means of RP-HPLC. The proinsulin-hirudin fusion protein is eluted at an NaCl concentration of about 300 mM. Sufficiently pure fractions are combined, diluted with 0,1% TFA and applied to an RP column (PLRP -S 7.5 x 50 mm) by pumping. Elution is carried out using a gradient of 25–50% acetonitrile. Two groups of fractions are pooled. After removing the solvent, the material is freeze-dried. The purity of the material is checked by means of SDS polyacrylamide electrophoresis. The purified fusion protein is analyzed by mass spectrometry (ESI). The experimentally determined molecular weight of the fusion protein corresponds to its theoretically expected molecular weight after removal of the signal peptide.

25

Example 7: Determination of the disulfide bridge linkage

The fusion protein is digested with trypsin and the fragments formed are analyzed by means of RP-HPLC and subsequently by means of mass spectrometry. A fragment which is recognized as de-(B30) insulin, due to its mass of 5706 dt, is successfully identified. This product is subjected to *S. aureus* V8 protease digestion. RP-HPLC analysis shows the expected peptide pattern.

30

Trypsin cleavage is carried out as follows:

The freeze-dried fusion protein is dissolved in 50 mM Tris-HCl pH 8 (1 mg/ml), and trypsin (1 µg per mg of fusion protein) is added. Trypsin is inactivated at pH 3 at the end of the reaction.

- 5 The *S. aureus* digestion is carried out as follows:

The isolated de-(B30) insulin is dissolved in water at pH 8, *S. aureus* protease (1/50 of the amount of insulin) is added, and the mixture is incubated at 37°C for 5 hours and then at room temperature overnight.

10

Example 8: Purification of insulin

- 15 In contrast to most other polypeptides found in the supernatant due to either spontaneous lysis of host cells or secretion, the fusion protein is surprisingly not precipitated at pH 2.5–3. The culture medium is therefore acidified appropriately and then, after completion of the precipitation, the precipitate and the cells are removed by centrifugation or by microfiltration and concentrated.

- 20 Subsequently, the medium is adjusted to pH 6.8 and the fusion protein content is determined in parallel by analytical HPLC measurement. The determination is followed by adding trypsin to the supernatant so that trypsin is at approx. 1 µg per 1–1.5 mg of fusion protein. After incubation at room temperature for approx. 4 hours, purification is carried out by cation exchange chromatography at pH 3.5 in the
25 presence of 2-propanol. Elution is carried out in the buffer by applying a gradient of from 0.15 to 0.45 M.

- Di-Arg-insulin is eluted at approx. 0.3 M. After 1:1 dilution, di-Arg-insulin is precipitated from the insulin-containing fractions at pH 6.8 with the addition of a 10%
30 strength ZnCl₂ solution. Insulin is filtered off and then dissolved in 0.05 M Tris-HCl (pH 8.5) resulting in a 2 mg/ml solution:

Then the amount of approximately 1 unit of carboxypeptidase B per 100ml solution is added and the reaction is carried out with gentle stirring. The pH is then adjusted to pH 5.5 with citric acid, and insulin is crystallized in the presence of ZnCl₂. The

crystals are removed, dissolved and, after purification by RP-HPLC, insulin is purified again by crystallization.

5

Example 9: Processing of the fusion protein directly in the culture medium

- 10 At the end of the expression period, the culture medium is adjusted to pH 6.8 and trypsin is then added with stirring so that a final concentration of 4–8 mg per liter is established. After incubation for approx. 4 hours, the fermentation broth treated in this way is adjusted to pH 2.5–3. After 1-6 hours of precipitation, the pH is raised to 3.5, and the di-Arg-insulin formed is purified via cation exchange chromatography in
- 15 the presence of 30% 2-propanol. Elution is carried out by means of an NaCl gradient of 0.05–0.5 M salt. The product-containing fractions are diluted 1:1 with H₂O and then ZnCl₂ is added, so that a 0.1% strength ZnCl₂ solution is formed. Di-Arg-insulin precipitates at pH 6.8 and by way of example is converted to insulin according to example 8.

Patent claim:

1. A DNA coding for a fusion protein of the form

5 -F-As_m-R_n-Y-

where

F is a DNA sequence coding for an amino acid sequence which allows secretion of a protein Y into a fermentation medium,

10 As is a chemical bond or a DNA sequence coding for an amino acid encodable by genetic engineering,

m is an integer from 0 - 10,

R is a chemical bond or an arginine codon,

n is 0 or 1, and

15 Y is a DNA sequence coding for a protein of interest which, correctly folded, is part of the fusion protein in the fermentation medium.

Abstract

5 Fusion protein for the secretion of a protein of interest into bacterial supernatants

The invention relates to fusion proteins comprising a fusion part and a protein of interest, the combination of the two proteins leading to the fusion protein being secreted into the supernatant of a bacterial host and the protein of interest being

10 present in its correct three-dimensional structure.



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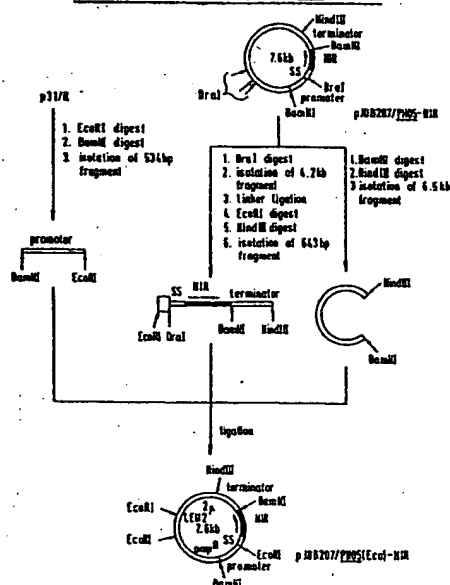
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(54) Production of thrombin inhibitors.

(57) A method for the production and secretion of proteins with hirudin activity in an eukaryotic host organism is provided. There are also provided hybrid vectors comprising a DNA sequence encoding a signal peptide upstream of and in reading frame with the structural gene for desulphatohirudin, and eukaryotic host organisms transformed with said hybrid vectors.

CONSTRUCTION OF PLASMID pJ08207/PH05(Eco)-HIR



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Production of thrombin inhibitors

The invention pertains to the field of recombinant DNA technology and concerns a method for the preparation of the thrombin inhibitor desulphathirudin with the aid of genetically engineered eukaryotic cells, said genetically engineered eukaryotic cells, hybrid vectors carrying a gene for desulphathirudin and methods for the preparation of said eukaryotic cells and said hybrid vectors.

Hirudin is an anticoagulant agent that occurs naturally in leeches (Hirudo medicinalis). Hirudin is not a single protein species but consists of at least three components designated hirudin variant 1 (HV1), hirudin variant 2 (HV2) - (cf. European Patent Application No. 158,564) and "des-(Val)₂-hirudin" (cf. European Patent Application No. 158,986). The variants differ in structure from each other by a number of amino acids (especially, the N-terminal sequence of HV1 is Val-Val-Tyr, that of HV2 is Ile-Thr-Tyr and that of "des-(Val)₂-hirudin" is Thr-Tyr) but have an accumulation of hydrophobic amino acids at the N-terminus and of polar amino acids at the C-terminus, a tyrosine residue (Tyr⁶³) present as sulphate monoester, three disulphide bridges and the anticoagulant activity in common.

Hirudin, with a K_i -value (complex dissociation constant) of 6×10^{-11} M, is the strongest thrombin inhibitor known and is characterised by a specific affinity to thrombin. Other enzymes of the blood coagulation cascade are not inhibited by hirudin. In contrast to heparin which is the preferred anticoagulant in conventional anticoagulation therapy, hirudin exerts its inhibiting action directly on thrombin and, unlike the former, does not act through antithrombin III. The only pharmacologically detectable

effect of purified hirudin is the inhibition of blood coagulation and the prophylaxis of thrombosis. No effect on heart rate, respiration, blood pressure, thrombocyte count, fibrinogen and haemoglobin could be observed when administered intravenously to dogs, even in high doses. In tests on rats, pigs and dogs, hirudin has proved effective in experimental thrombosis (induced either by stasis or by the injection of thrombin), in endotoxin shock, and also in DIC (disseminated intravascular coagulation). Whenever direct comparison tests have been carried out, hirudin has proved to be superior to heparin. Furthermore, hirudin has an extremely low toxicity, is non antigenic and shows an almost complete clearance via the kidneys in a biologically active form.

Although long known, hirudin has not as yet achieved the broad therapeutic use that might be expected on the basis of its excellent biological properties. Its extremely limited availability is a serious drawback which stands in the way of its widespread use in medicine. Up to now, hirudin preparations have been obtainable essentially from natural material (leech extracts), which is expensive and difficult to obtain, and employing time-consuming and costly isolation and purification processes [cf. P. Walsmann et al., *Pharmazie* 36, 653 (1981); European Patent Application No. 158,986]. In view of the relatively long sequence of 65 amino acids also the conventional peptide synthesis offers little hope of success for economic reasons. New methods must therefore be applied for the manufacture of adequate amounts of hirudin that render possible detailed clinical tests of its therapeutic potential and its broad therapeutic use in anticoagulation therapy.

Such methods are offered especially by recombinant DNA technology. By means of this technology it is possible to manufacture the most varied physiologically active poly-

peptides by culturing correspondingly genetically modified host organisms. In this context, it has to be mentioned that hirudin is presumably produced by leeches via a desulphatohirudin precursor which is post-translationally sulphated. It is expected that hosts other than leech lack the specific sulphate-transferring enzyme system and will therefore produce desulphatohirudins rather than hirudins. However, the biological activity is not injured by the absence of the sulphate group as is evidenced by the desulphatohirudin protein obtained by enzymatic removal of the sulphate group from the phenolic hydroxy group of the Tyr 63 residue of the corresponding hirudin protein (cf. European Patent Application No. 142,860).

In the recently published European Patent Application No. 158,564 the production of desulphatohirudin variants 1 and 2 and of analogues thereof by means of E.coli cells transformed with plasmids containing the structural genes for the respective desulphatohirudins is disclosed. The anticoagulant activity measured in cell extracts of cultured E.coli cells and given in terms of anti-thrombin units ("ATU") amounts to 3,000-4,000 ATU/OD₆₀₀/l culture which corresponds to a concentration of 0.2 mg hirudin/OD/1 (based upon an estimated specific activity of 15,000-20,000 ATU/mg of pure hirudin).

Presumably the poor yield in hirudin activity obtained from transformed E.coli cells is attributable to an accumulation of most of the hirudin protein in an inactive form in the cytoplasm due to incorrect folding of the molecule. The correct folding depends on the formation of three disulphide bridges within the hirudin molecule which are essential for enzyme activity (cf. P. Walsmann et.al., supra). Other naturally secreted mammalian proteins, such as bovine growth hormone, human tissue plasminogen activator and human

β -interferon are likewise essentially inactive when produced and accumulated in the cytoplasm of microorganisms [cf. R.A. Smith et al., Science 229, 1219 (1985)]. It is apparent that the secretion pathway may favor disulphide bond formation since most proteins containing disulphide bridges are extracellular. There are other features that make secretion most desirable:

Secreted proteins are generally easier to detect and to purify than intracellularly accumulated products; secretion of desired products into the medium avoids the necessity of breaking up the host organisms in order to recover the product;

some heterologous proteins may have a toxic effect on the host organism. When secreted they are less likely to interfere with normal cellular functions;

secreted proteins are less likely to be digested by proteolytic enzymes than intracellularly accumulated proteins which are attacked by these enzymes upon disintegration of the cells.

Most secreted proteins are encoded on the DNA as preproteins with a signal peptide as an appended amino terminal extension of the mature amino acid sequence. The signal peptide plays an essential role during cotranslational insertion of the protein into the membranes of the endoplasmatic reticulum (ER). A signal peptidase cleaves the signal peptide in an early event on the luminal side of the ER. Further transport to the outer cell membrane involves Golgi and secretory vesicles. The protein is either secreted into the periplasmic space (e.g. acid phosphatase, invertase) or into the culture medium (e.g. α factor, killer toxin).

Since all eukaryotes seem to share the mechanisms for the expression of genetic information and for sorting the expressed proteins, the expression of eukaryotic genes proceeds with greater efficiency in an eukaryotic host than

in E.coli. Among the eukaryotic organisms yeast is the easiest to handle and to cultivate. A number of heterologous proteins have been expressed successfully in yeast. However, it has not been possible so far to define essential features of a protein - except for an appended signal peptide - that would allow efficient secretion into the medium. Therefore, it is not possible to make reliable predictions whether or not a protein will be secreted. Thus, while 90 % of total glucoamylase produced by transformed yeast (containing the genetic information for pre-glucoamylase) is secreted into the medium (PCT-Patent Application No. 84/2921) and high titers of epidermal growth factor (EGF) are found in the medium of cultured yeast containing the EGF gene with the appended α factor signal peptide (European Patent Application No. 116,201), only minor amounts or traces of β -endorphin (α -factor signal peptide, PCT-Patent Application No. 84/4330), leukocyte interferon A (invertase signal peptide, European Patent Application No. 127,304), human γ -interferon, human serum albumin, bovine interferons α 1 and α 2, tissue plasminogen activator, rennin and human insulin-like growth factor (α -factor signal peptide, European Patent Application No. 123,544), leukocyte interferons D and A, γ -interferon and human growth hormone (MGH) (interferon and MGH signal peptides, respectively, European Patent Application No. 88,632) are detectable in the medium, and no secretion of Pseudomonas carboxypeptidase G_2 (CPG₂) [G_2 (CPG₂) signal peptide, European Patent Application No. 121,352] and tissue plasminogen activator (PHO5 signal peptide, European Patent Application No. 143,081) by transformed yeast into the medium is observed.

Accordingly, whether or not a given protein having an appended signal peptide is secreted by yeast is unpredictable and depends above all on the selected protein.

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Hence, it was uncertain, whether a selected protein, such as hirudin, having an appended signal peptide would be secreted, at least to a certain degree, by transformed host organisms, such as yeast.

Surprisingly, it has now been found that proteins with hirudin activity are secreted into the medium by eukaryotic host organisms which contain a DNA comprising a DNA sequence encoding a signal peptide upstream of and in reading frame with the structural gene for desulphato-hirudin.

It is an object of the present invention to provide a method for the production and secretion of proteins with hirudin activity in an eukaryotic host organism.

It is a further object of the invention to provide hybrid vectors comprising a DNA sequence encoding a signal peptide upstream of and in reading frame with the structural gene for desulphatohirudin, and eukaryotic host organisms transformed with said hybrid vectors.

Detailed description of the invention

1. Process for the production of desulphatohirudin

The invention concerns a method for the production of proteins with hirudin activity comprising culturing under appropriate nutrient conditions an eukaryotic host organism transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and isolating the proteins with hirudin

activity from the culture medium and, if desired, isolating optionally additional protein with hirudin activity which is cell associated from the cell interior, and, if desired, converting a resulting protein with hirudin activity into a different protein with hirudin activity.

The term "DNA sequence coding for mature desulphatohirudin" is intended to embrace all structural genes coding for mature proteins having hirudin activity which are known to exist in and/or can be isolated from the leech genome, such as the structural genes for mature desulphatohirudin variants HV1, HV2, PA and for des-(Val)₂-desulphatohirudin (unless the latter is a mere artefact of expression of the HV1 gene). The term "mature desulphatohirudin" refers to desulphatohirudins which are devoid of any pre- and pro-sequences.

There are to be understood as "proteins with hirudin activity" those proteins obtained from the secreting host cells that have a thrombin-inhibiting action and a positive reaction with anti-hirudin antibodies and that have the primary structure of a desulphatohirudin, as well as modified, such as sulphated, derivatives thereof, e.g. the corresponding hirudins, and shortened derivatives thereof, such as desulphatohirudins lacking one to seven amino acids at the C-terminus.

Such proteins are especially those having the formulae

X Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu Gln

|
R

(I),

in which X represents the dipeptide residue Val-Val- (HV1) or Thr (Des-(Val)₂-hirudin) and R represents the phenolic hydroxy group of Tyr or a group of the formula

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-O-SO₃H, and the derivatives lacking the C-terminal amino acid Gln, the C-terminal dipeptide -Leu-Gln, the C-terminal tripeptide -Tyr(R)-Leu-Gln or the C-terminal tetrapeptide -Glu-Tyr(R)-Leu-Gln, or

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
 Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
 Ile Leu Gly Ser Asn Gly Lys Gly Asn Gln Cys Val Thr Gly
 Glu Gly Thr Pro Asn Pro Glu Ser His Asn Asn Gly Asp Phe
 Glu Glu Ile Pro Glu Glu Tyr Leu Gln
 |
 R

(II)

(HV2) in which R has the meanings given above, or another hirudin variant named hirudin PA which is produced by leech (J. Dodt, Thesis, University of Munich, FRG, 1984) and has the formula

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
 Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
 Ile Leu Gly Ser Gln Gly Lys Asp Asn Gln Cys Val Thr Gly
 Glu Gly Thr Pro Lys Pro Gln Ser His Asn Gln Gly Asp Phe
 Glu Pro Ile Pro Glu Asp Ala Tyr Asp Glu
 |
 R

(III)

(PA) in which R has the meanings given above.

The preferred protein with hirudin activity is that of formula I in which X represents the residue Val-Val- and R represents the phenolic hydroxy group of Tyr, and the derivatives thereof that are shortened at the C-terminus by one to seven, especially one to four amino acids.

Suitable eukaryotic host organisms are cells of higher organisms, especially mammals, in particular established human or animal cell lines, such as VERO or HeLa cells, or the Chinese hamster ovary (CHO) cell line, and yeast, such as Saccharomyces cerevisiae. The preferred host organism according to the present invention is yeast, especially strains of Saccharomyces cerevisiae.

The eukaryotic host organism, especially yeast, containing the above DNA sequences is cultured using methods known in the art.

Thus, transformed yeast strains according to the invention are cultured in a liquid medium containing assimilable sources of carbon, nitrogen and inorganic salts.

Various carbon sources are usable. Examples of preferred carbon sources are assimilable carbohydrates, such as glucose, maltose, mannitol or lactose, or an acetate such as sodium acetate, which can be used either alone or in suitable mixtures. Suitable nitrogen sources include, for example, amino acids, such as casamino acids, peptides and proteins and their degradation products, such as tryptone, peptone or meat extracts, furthermore yeast extract, malt extract, corn steep liquor, as well as ammonium salts, such as ammonium chloride, sulphate or nitrate which can be used either alone or in suitable mixtures. Inorganic salts which may be used include, for example, sulphates, chlorides, phosphates and carbonates of sodium, potassium, magnesium and calcium. Additionally, the nutrient medium may also contain growth promoting substances. Substances which promote growth include, for example, trace elements, such as iron, zinc, manganese and the like, or individual amino acids.

- 10 -

To a varying extent, yeast cells transformed with autonomously replicating plasmids, for example, plasmids containing yeast 2 μ plasmid DNA (infra) tend to lose the introduced hybrid plasmid. For this reason, such yeast cells have to be grown under selective conditions, i.e. conditions which require the expression of a plasmid-encoded gene for growth. Most selective markers currently in use and present in the hybrid vectors according to the invention (infra) are genes coding for enzymes of amino acid or purine biosynthesis. This makes it necessary to use synthetic minimal media deficient in the corresponding amino acid or purine base. However, genes conferring resistance to an appropriate biocide may be used as well [e.g. genes conferring resistance to cycloheximide, to the amino-glycoside G 418, to a heavy metal, or the like]. Yeast cells transformed with vectors containing antibiotic resistance genes are grown in complex media containing the corresponding antibiotic whereby faster growth rates and higher cell densities are reached.

Yeast cells transformed with DNA integrating into a chromosome do not require selective growth conditions. These transformed cells are sufficiently stable to allow growth without selective pressure. These cells are advantageously grown in complex media.

Yeast cells containing hybrid plasmids with a constitutive promoter (e.g. ADHI, GAPDH) express the desulphatohirudin gene controlled by said promoter without induction. However, if the desulphatohirudin gene is under the control of a regulated promoter (e.g. PGK or PHO5) the composition of the growth medium has to be adapted in order

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to obtain maximum levels of mRNA transcripts, i.e. when using the PHO5 promoter the growth medium must contain a low concentration of inorganic phosphate for derepression of this promoter.

The cultivation is carried out by employing conventional techniques. The culturing conditions, such as temperature, pH of the medium and fermentation time are selected in such a way that maximal levels of desulphatohirudin are produced. A chosen yeast strain is preferably grown under aerobic conditions in submerged culture with shaking or stirring at a temperature of about 25° to 35°C, preferably at about 30°C, at a pH value of from 4 to 8, for example at approximately pH 7, and for about 4 to 20 hours, preferably until maximum yields of proteins are reached.

It is surprisingly found that, irrespective of the host organism and the signal peptide used, most of the produced hirudin compounds are secreted into the culture medium whereas only a minor part remains cell associated. The precise ratio (secreted compounds/cell associated compounds) depends on the fermentation conditions and the recovery process applied. In general it amounts to about or more than 9:1. Accordingly, secreted hirudin is always strongly dominating.

The hirudin compounds can be isolated from the culture medium by conventional means. For example, the first step consists usually in separating the cells from the culture fluid by means of centrifugation. The resulting supernatant can be enriched for hirudin compounds by treatment with polyethyleneimine so as to remove most of the non-proteinaceous material, and precipitation of the proteins by saturating the solution with ammonium sulphate or by trichloroacetic acid. Host proteins, if present,

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can also be precipitated by means of acidification with acetic acid (for example 0.1%, pH 4-5). A further enrichment of hirudin compounds can be achieved by extracting the acetic acid supernatant with n-butanol. Other purification steps include, for example, desalination, chromatographic processes, such as ion exchange chromatography, gel filtration chromatography, partition chromatography, HPLC, reversed phase HPLC and the like. The separation of the constituents of the mixture is also effected by dialysis, according to charge by means of gel electrophoresis or carrier-free electrophoresis, according to molecular size by means of a suitable Sephadex column, by affinity chromatography, for example with antibodies, especially monoclonal antibodies, or with thrombin coupled to a suitable carrier for affinity chromatography, or by other processes, especially those known from the literature.

If it is desired to isolate any additional hirudin compounds which are cell associated, i.e. which have accumulated intracellularly or in the periplasmic space, some supplementary purification steps are required. Thus, in case the hirudin proteins have accumulated within the cells, the first step for the recovery of the desired proteins consists in liberating the proteins from the cell interior. In most procedures the cell wall is first removed by enzymatic digestion with glucosidases (infra). Subsequently, the resulting spheroplasts are treated with detergents, such as Triton. Alternatively, mechanical forces, such as shearing forces (for example X-press, French-press) or shaking with glass beads, are suitable for breaking cells. In the case where the hirudin compounds are secreted by the host, especially yeast, cells into the periplasmic space,

a simplified protocol can be used: The protein is recovered without cell lysis by enzymatic removal of the cell wall or by treatment with chemical agents, e.g. thiol reagents or EDTA, which give rise to cell wall damages permitting the produced protein to be released.

It is surprisingly found that apart from the desulphato-hirudin compounds corresponding to the "DNA sequence coding for mature desulphatohirudin" some other desulphatohirudin compounds can be isolated from the culture broth which differ from the expected compounds by the absence of one to four amino acids at the C-terminus. Thus, culturing of yeast cells carrying the gene coding for desulphatohirudin variant HV 1 yields this variant as well as, in lower yields, desulphatohirudin compounds lacking the C-terminal amino acid Gln [Des-(Gln⁶⁵)-desulphatohirudin], the C-terminal dipeptide residue - Leu-Gln [Des-(Leu⁶⁴, Gln⁶⁵)-desulphatohirudin], the C-terminal tripeptide residue -Tyr-Leu-Gln [Des-(Tyr⁶³, Leu⁶⁴, Gln⁶⁵)-desulphatohirudin] and the C-terminal tetrapeptide residue -Glu-Tyr-Leu-Gln [Des-(Glu⁶², Tyr⁶³, Leu⁶⁴, Gln⁶⁵)-desulphatohirudin], respectively. These compounds may have been formed by (partial) proteolytical degradation of the primary expression product desulphatohirudin and have been identified in all culture broths, irrespective of the yeast host used and the fermentation conditions applied.

Des-(Gln⁶⁵)-desulphatohirudin, Des-(Tyr⁶³, Leu⁶⁴, Gln⁶⁵)-desulphatohirudin and Des-(Glu⁶², Tyr⁶³, Leu⁶⁴, Gln⁶⁵)-desulphatohirudin are novel and are also objects of the present invention.

The test with anti-hirudin or anti-desulphatohirudin antibodies (for example, monoclonal antibodies obtainable from hybridoma cells), the thrombin test [M.U. Bergmeyer (ed.), Methods in Enzymatic Analysis, Vol. II, p. 314-316, Verlag Chemie, Weinheim (FRG) 1983] or the blood coagulation test [F. Markwardt et al., Thromb. Haemost. 47, 226 (1982)] can be used to detect the hirudin activity.

Hirudin compounds obtainable in accordance with the process can be converted in a manner known per se into different hirudin compounds.

Thus, for example, compounds of the formula I to III in which R represents the phenolic hydroxy group of Tyr can be converted by reaction with a sulphate, such as disodium sulphate, and a tyrosine sulphotransferase, obtainable, for example, from leech cells, into compounds of the formula I to III in which R represents a group of the formula $-\text{OSO}_3\text{H}$.

It is also possible to convert a desulphatohirudin compound obtained, for example desulphatohirudin variant HV 1, into a derivative thereof that lacks one to seven amino acids at the C-terminus, for example by the action of a suitable carboxypeptidase, such as carboxypeptidase Y.

The transformed eukaryotic host organisms according to the invention can be prepared by recombinant DNA techniques comprising the steps of

- preparing a DNA construct containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals,
- preparing a hybrid vector comprising said DNA construct,
- transforming a suitable eukaryotic host organism with the created hybrid vector,
- and selecting transformed host cells from untransformed host cells.

2. DNA constructs comprising the coding regions of a signal peptide and of desulphatohirudin

The invention concerns a DNA construct containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals.

Several expression control sequences can be used for regulation of expression of the DNA sequences coding for the signal peptide and desulfatohirudin. In particular, expression control sequences of highly expressed genes of the host to be transformed are used.

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For use in mammalian cells, the expression control sequences are preferably derived from *vira*. For example, suitable expression control sequences for use in mammalian cells are the early and late promoters of Simian Virus 40 (SV40), the vaccinia promoter, HTLV promoter or promoters derived from polyoma or Adenovirus 2.

Expression control sequences for yeast which are the most preferred host organisms according to the present invention are derived from the genomic DNA of yeast, especially of *Saccharomyces cerevisiae*. Preferably, the expression control sequence of a highly expressed yeast gene is used for the expression of desulphatohirudin. Thus the promoter of the TRP1 gene, the ADHI or ADHII gene, acid phosphatase (PHO5) gene, isocytochrome c gene, or a promoter of the enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase and glucokinase genes, or a promoter of the yeast mating pheromone genes coding for the a- or α -factor, can be used. It is also possible to use hybrid promoters comprising upstream activation sequences (UAS) of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene, for example a hybrid promoter including the UAS(s) of the yeast PHO5 gene and downstream promoter elements including a functional TATA box of the yeast GAPDH gene. Preferred vectors of the present invention contain promoters with transcriptional control. Promoters of this type, e.g. the promoter of the PHO5 gene and PHO5-GAPDH hybrid promoters, can be turned on or off by variation of the growth conditions. For example, the PHO5 promoter can be repressed or derepressed at will,

solely by increasing or decreasing the concentration of inorganic phosphate in the medium. A further preferred promoter according to the invention is the promoter of the GAPDH gene, especially a functional fragment thereof starting at nucleotide -300 to -180, in particular at nucleotide -263 or -199, and ending at nucleotide -5 of the GAPDH gene.

The DNA sequence encoding a signal peptide ("signal sequence") is preferably derived from eukaryotic, for example yeast, genes coding for polypeptides which are ordinarily secreted. Suitable signal sequences are, for example, the hirudin signal sequence obtainable from genomic leech DNA, yeast signal sequences, such as the signal and prepro sequences of the yeast invertase, α -factor, α -factor, pheromone peptidase, "killer toxin" and repressible acid phosphatase (PHO5) genes and the glucomylase signal sequence from Aspergillus awamori. Alternatively, fused signal sequences may be constructed by ligating part of the signal sequence (if present) of the gene naturally linked to the promoter used, with part of the hirudin signal sequence. Those combinations are favoured which allow a precise cleavage between the signal sequence and the mature desulphatohirudin amino acid sequence. Additional sequences, such as pro- or spacer-sequences which may or may not carry specific processing signals can also be included in the constructions to facilitate accurate processing of precursor molecules. Alternatively fused proteins can be generated containing internal processing signals which allow proper maturation in vivo or in vitro. For example, the processing signals contain a Lys-Arg residue, which is recognized by a yeast endopeptidase located in the Golgi membranes. The preferred signal sequences according to the present invention are those of the yeast PHO5 gene coding for a signal peptide having the formula

Met Phe Lys Ser Val Val Tyr Ser Ile Leu Ala Ala Ser Leu Ala
Asn Ala,

and of the yeast invertase gene coding for a signal peptide
having the formula

Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala Gly Phe Ala
Ala Lys Ile Ser Ala

The DNA sequence coding for mature desulphatohirudin is especially selected from the structural genes for desulphatohirudins specified above. The preferred DNA sequence codes for desulphatohirudin variant 1 (HV1) and has the formula

GTTGTTTACACCGACTGCACCGAATCTGGTCAGAACCTGTGCCTGTGCGAAGGT
CAACAAATGTGGCTGACGTGGCTTAGACCAGTCTTGGACACGGACACGCTTCCA

TCTAACGTTTGCGGTCAGGGTAACAAATGCATCCTGGGTTCTGACGGTGAAAAAACCAG
AGATTGCAAACGCCAGTCCCATTGTTTACGTAGGACCCAAGACTGCCACTTTTTTTGGTC

TGCGTTACCGGTGAAGGTACCCGAAACCGCAGTCTCACAACGACGGTGACTTCGAA
ACGCAATGGCCACTTCCATGGGGCTTTGGCGTCAGAGTGTTGCTGCCACTGAAGCTT

GAAATCCCGGAAGAATACCTGCAG
CTTTAGGGCCTTCTTATGGACGTC.

A DNA sequence containing eukaryotic transcription termination signals is preferably the 3' flanking sequence of an eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences are for example those of the gene naturally linked to the expression control sequence used. In case yeast is used as the host organism the preferred flanking sequences are those of the yeast PHO5 gene. Preferably, the DNA construct according to the present invention is provided at both ends with synthetic deoxynucleotide linkers which allow insertion and cloning of the construct in a cloning vector.

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The eukaryotic expression control sequence, the DNA sequence coding for the signal peptide, the DNA sequence coding for mature desulphatohirudin and the eukaryotic transcription termination signals are operably linked to each other, i.e. they are juxtaposed in such a manner that their normal functions are maintained. Thus, the array is such that the expression control sequence effects proper expression of the signal peptide-desulphatohirudin gene complex, the transcription termination signals effect proper termination of transcription and polyadenylation and the signal sequence is linked to the desulphatohirudin gene in such a manner that secretion of desulphatohirudin occurs. Accordingly, if the expression control sequence and the signal sequence are derived from different genes, the expression control sequence is preferably joined to the signal sequence between the major mRNA start and the ATG of the gene naturally linked to the expression control sequence. The signal sequence should have its own ATG for translation initiation. The junction of these sequences is preferable effected by means of synthetic oligodeoxynucleotide linkers which may carry the recognition sequence of an endonuclease. On the other hand, the last codon of the signal sequence is directly linked to the first codon of the gene for desulphatohirudin.

The DNA constructs according to the invention may be prepared by methods known in the art, for example, by linking an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for desulphatohirudin, and a DNA sequence containing eukaryotic transcription termination signals in such a way that proper

expression of the DNA segment and secretion of the produced desulphatohirudin is effected in an eukaryotic host organism.

Linkage of the various DNA sequences to yield the DNA constructs according to the invention may be effected by blunt end ligation or via suitable common restriction sites or synthetic linker molecules taking special care to a correct junction so that the normal functions of these DNA sequences are maintained. Thus, the signal sequence and the desulphatohirudin gene can be fused by blunt end ligation. Another approach for creating the correct junction of the signal sequence and the desulphatohirudin gene consists in restricting, if possible, the signal sequence near its 3' terminus and the desulphatohirudin near its 5' terminus so that each sequence lacks a predetermined number of base pairs. A synthetic oligodeoxynucleotide linker can then be constructed in such a way that, when joining the restricted signal sequence and the restricted desulphatohirudin gene via the connecting oligodeoxynucleotide, the missing base pairs are restored and the desulphatohirudin gene is in the proper reading frame relative to the signal sequence.

The DNA sequence coding for desulphatohirudin can be isolated from genomic leech DNA or a complementary double-stranded desulphatohirudin DNA (desulphatohirudin ds cDNA) is produced from desulphatohirudin mRNA, or a gene coding for the amino acid sequence of desulphatohirudin is produced by means of chemical and enzymatic processes. Genomic desulphatohirudin DNA and desulphatohirudin ds cDNA are obtained, for example, according to methods that are known per se. For example, genomic desulphatohirudin DNA is obtained from a leech gene bank that contains the desulphatohirudin gene by cloning the leech DNA fragments in a microorganism and identifying clones that contain desul-

phathohirudin DNA, for example by colony hybridisation using a radioactively labelled desulphathohirudin DNA-specific oligodeoxynucleotide that contains at least 15, and preferably from 15 to 30, deoxynucleotides. The resulting DNA fragments as a rule contain in addition to the desulphathohirudin gene other undesired DNA constituents that can be removed by treatment with suitable exo- or endo-nucleases.

Double-stranded desulphathohirudin cDNA can be produced, for example, by isolating mRNA from suitable leech cells, which are preferably induced to form hirudin, enriching the desulphathohirudin mRNA in the resulting mRNA mixture in a manner known per se, using this mRNA as a template for the preparation of single-stranded cDNA, synthesising from this, with the aid of an RNA-dependent DNA polymerase, ds cDNA, and cloning the latter into a suitable vector. Clones that contain desulphathohirudin cDNA are identified, for example in the manner described above, by colony hybridisation using a radioactively labelled, desulphathohirudin DNA-specific oligodeoxynucleotide.

The desulphathohirudin gene can also be produced by chemical synthesis. The process is characterised in that segments of the coding and of the complementary strand of said gene are chemically synthesised and resulting segments are converted enzymatically into a structural gene for desulphathohirudin.

The chemical synthesis of DNA is well-known in the art and makes use of conventional techniques. Appropriate techniques have been compiled by S.A. Narang [Tetrahedron 39, 3 (1983)]. In particular, the methods described in European Patent Application No. 146,785 may be used and are herein incorporated by reference.

In an analogous manner, the signal sequence can be prepared by chemical synthesis or is isolated from chromosomal DNA of a suitable eukaryotic organism.

3. Hybrid vectors containing DNA constructs with the coding regions of a signal peptide and of desulphatohirudin

According to the present invention there is further provided a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals.

The hybrid vectors according to the invention are selected from the group consisting of a hybrid plasmid and a linear DNA vector and are further selected depending on the host organism envisaged for transformation.

The invention relates especially to a linear DNA vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, said expression control sequence and said sequence containing transcription termination signals being derived from the same eukaryotic gene. Especially, the invention relates to a linear DNA vector comprising the yeast PHO5 promoter, the above DNA segment and the 3' flanking region of PHO5.

By virtue of the homologous 3' and 5' flanking sequences the whole linear DNA vector including the coding regions for the signal peptide and desulphatohirudin is stably integrated into the respective host chromosome, i.e. in the case of the yeast PHO5 promoter and 3' flanking sequences of the yeast PHO5 gene at the PHO5 locus in yeast chromosome II.

The invention relates also especially to hybrid plasmids which apart from the expression control sequence, the above DNA segment and the sequence containing transcription termination signals contain additional DNA sequences which are inessential or less important for the function of the promoter, i.e. for the expression of the desulphatohirudin gene, but which perform important functions, for example, in the propagation of the cells transformed with said hybrid plasmids. The additional DNA sequences may be derived from prokaryotic and/or eukaryotic cells and may include chromosomal and/or extra-chromosomal DNA sequences. For example, the additional DNA sequences may stem from (or consist of) plasmid DNA, such as bacterial or eukaryotic plasmid DNA, viral DNA and/or chromosomal DNA, such as bacterial, yeast or higher eukaryotic chromosomal DNA. Preferred hybrid plasmids contain additional DNA sequences derived from bacterial plasmids, especially Escherichia coli plasmid pBR322 or related plasmids, bacteriophage λ , yeast 2 μ plasmid, and/or yeast chromosomal DNA.

In the preferred hybrid plasmids according to the invention, the additional DNA sequences carry a yeast replication origin and a selective genetic marker for yeast. Hybrid plasmids containing a yeast replication origin, e.g. an

autonomously replicating segment(ars), are extrachromosomally maintained within the yeast cells after transformation and are autonomously replicated upon mitosis.

Hybrid plasmids containing sequences homologous to yeast 2 μ plasmid DNA can be used as well. These hybrid plasmids will get integrated by recombination into 2 μ plasmids already present within the cell or will replicate autonomously.

As to the selective gene marker for yeast, any marker gene can be used which facilitates the selection for transformants due to the phenotypic expression of the marker. Suitable markers for yeast are particularly those expressing antibiotic resistance or, in the case of auxotrophic yeast mutants, genes which complement host lesions. Corresponding genes confer, for example, resistance to the antibiotic cycloheximide or provide for prototrophy in an auxotrophic yeast mutant, for example the URA1, URA3, ARG4, LEU2, HIS4, HIS3, TRP5 or TRP1 gene.

Advantageously, the additional DNA sequences which are present in the hybrid plasmids according to the invention also include a replication origin and a selective genetic marker for a bacterial host, especially Escherichia coli. There are useful features which are associated with the presence of an E.coli replication origin and an E.coli marker in a yeast hybrid plasmid. Firstly, large amounts of hybrid plasmid DNA can be obtained by growth and amplification in E.coli and, secondly, the construction of hybrid plasmids is conveniently done in E.coli making use of the whole repertoire of cloning technology based on E.coli. E.coli plasmids, such as pBR322 and the like, contain both E.coli replication origin and E.coli genetic markers conferring resistance to antibiotics, for example

tetracycline and ampicillin, and are advantageously employed as part of the yeast hybrid vectors.

The additional DNA sequences which contain, for example, replication origin and genetic markers for yeast and a bacterial host (see above) are hereinafter referred to as "vector DNA" which together with the above DNA construct, containing inter alia the expression control sequence and the desulphatohirudin gene, is forming a hybrid plasmid according to the invention.

The hybrid vectors according to the invention may contain one or multiple DNA inserts each comprising, inter alia, the expression control sequence, the DNA sequence encoding the signal peptide and the DNA sequence coding for mature desulphatohirudin. If the hybrid vectors contain multiple DNA inserts, preferably 2 to 4 DNA inserts, these can be present in a tandem array or at different locations of the hybrid vector. Preferred hybrid vectors contain one DNA insert or DNA inserts in a tandem array. The DNA inserts are especially head to tail arranged.

The hybrid plasmids according to the invention are prepared by methods known in the art. The process for the preparation of the hybrid vectors comprises introducing one or multiple DNA constructs containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, as such or introducing the components of said DNA constructs successively in the predetermined order into a vector DNA.

The construction of the hybrid plasmids according to the invention is performed applying conventional ligation techniques. The components of the plasmids are linked through common restriction sites and/or by means of synthetic linker molecules and/or by blunt end ligation.

The linear DNA vectors according to the invention correspond essentially to the DNA constructs specified in paragraph 2 and are prepared in an analogous manner.

4. Transformed eukaryotic host organisms

Another aspect of the invention involves eukaryotic host organisms transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and mutants thereof.

Suitable eukaryotic host organisms are especially those specified above, in particular yeast including species of the genera Kluyveromyces, Candida, Pichia, Yarrowia, Saccharomyces, Schizosaccharomyces, Torulopsis, and related genera (cf. J. Lodder, The Yeasts, Amsterdam 1971), especially strains of Saccharomyces cerevisiae.

The transformed eukaryotic host organisms are selected from an eukaryotic host organism transformed with a hybrid plasmid having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under

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transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and an eukaryotic host organism with said DNA insert(s) stably integrated in a host chromosome.

The process for the production of said transformed eukaryotic host cells comprises transforming eukaryotic host cells with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals.

The transformation of the eukaryotic host cells is accomplished by methods known in the art. For example, the transformation of yeast with the hybrid vectors may be accomplished according to the method described by Hinnen et al [Proc. Natl. Acad. Sci. USA 75, 1929 (1978)]. This method can be divided into three steps:

(1) Removal of the yeast cell wall or parts thereof using various preparations of glucosidases, such as snail gut juices (e.g. Glusulase[®] or Helicase[®]) or enzyme mixtures obtained from microorganisms (e.g. Zymolyase[®]) in osmotically stabilized solutions (e.g. 1M sorbitol).

(2) Treatment of the "naked" yeast cells (spheroplasts) with the DNA vector in the presence of PEG (polyethylene-glycol) and Ca^{2+} ions.

(3) Regeneration of the cell wall and selection of the transformed cells in a solid layer of agar. This rege-

neration is conveniently done by embedding the spheroplasts into agar. For example, molten agar (about 50°C) is mixed with the spheroplasts. Upon cooling the solution to yeast growth temperatures (about 30°C), a solid layer is obtained. This agar layer is to prevent rapid diffusion and loss of essential macromolecules from the spheroplasts and thereby facilitates regeneration of the cell wall. However, cell wall regeneration may also be obtained (although at lower efficiency) by plating the spheroplasts onto the surface of preformed agar layers.

Preferably, the regeneration agar is prepared in a way to allow regeneration and selection of transformed cells at the same time. Since yeast genes coding for enzymes of amino acid biosynthetic pathways are generally used as selective markers (supra), the generation is preferably performed in yeast minimal medium agar. If very high efficiencies of regeneration are required the following two step procedure is advantageous: (1) regeneration of the cell wall in a rich complex medium, and (2) selection of the transformed cells by replica plating the cell layer onto selective agar plates.

When the above linear DNA vector is used for transforming the eukaryotic host cells, transformation is preferably done in the presence of a second vector containing a selective marker for yeast. This cotransformation allows enrichment for those host cells which have taken up DNA that cannot be directly selected for. Since competent cells take up any type of DNA a high percentage of cells transformed with a selective vector will also harbor any additional DNA (such as the above linear DNA vector).

The transformed eukaryotic host organisms, especially the transformed yeast strains, containing the hybrid

plasmids according to the invention or having the linear DNA vector comprising the desulphatohirudin gene stably integrated in a host chromosome can be improved in production of desulphatohirudin by mutation and selection using methods known in the art. The mutation can be effected, for example, by U.V. irradiation or suitable chemical reagents. Especially preferred is the production of protease deficient mutants, especially yeast mutants, so as to avoid proteolytic degradation of the produced desulphatohirudin within the cells. Suitable mutants can be selected and isolated by conventional means.

The invention relates especially to the DNA constructs comprising the desulphatohirudin gene, the hybrid vectors, the transformed eukaryotic host organisms and to the processes for the preparation thereof as well as to the process for the production of proteins with hirudin activity as described in the Examples.

Brief description of the drawings

In the following experimental part various embodiments of the present invention are described with reference to the accompanying drawings in which:

Figures 1 and 2 are schematic diagrams showing the construction of plasmids pML300 and pML305, respectively.

Figure 3 depicts the construction of plasmid pHRil48 containing the trp promoter.

Figure 4 shows the construction of expression plasmid pML310.

Figure 5 is a schematic diagram showing the isolation of a DNA fragment coding for mature desulphatohirudin.

Figure 6 shows schematically the construction of an expression plasmid for secretion of desulphatohirudin.

Figure 7 shows schematically the construction of an expression plasmid for intracellular expression of desulphatohirudin.

Figure 8 depicts the DNA sequence of the BamHI-SalI restriction fragment of PHO5 including the PHO5 promoter region.

Figure 9 shows the DNA sequence of the promoter region of the GAPDH gene.

Figure 10 is a schematic diagram showing the construction of plasmid pGAPDH-EL.

Figure 11 depicts the sequences of the GAPDH promoter fragments present in plasmids pGAPDH-FL and pGAPDH-EL.

Figure 12 shows the construction of plasmid pJDB207/PHO5(Eco)-HIR.

Figure 13 shows the construction of plasmid pJDB207/PAPEL-HIR(UAS1 + UAS2).

Figure 14 depicts the construction of plasmid pJDB207/[GAPEL-HIR]D.

Figure 15 is a schematic diagram showing the construction of plasmid pJDB207/GAPFL-I-HIR.

The following abbreviations are used in the figures: p promoter, SS signal sequence, t terminator, L linker.

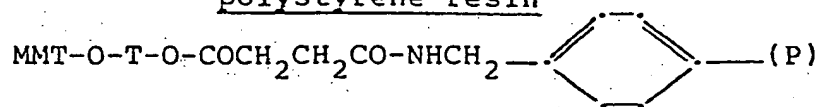
The following Examples serve to illustrate the present invention but should not be construed as a limitation thereof.

Experimental Part

The meanings of the abbreviations used in the Examples are as follows:

TNE solution that contains 100 mM NaCl, 50 mM tris•HCl
 pH 7.5 and 5 mM EDTA,
 SDS sodium dodecyl sulphate
 EDTA ethylenediaminetetraacetic acid
 DTT 1,4-dithiothreitol (1,4-dimercapto-2,3-butanediol)
 BSA bovine serum albumin
 EtBr ethidium bromide
 tris tris-(hydroxymethyl)-aminomethane
 tris•HCl tris monohydrochloride

Example 1: Manufacture of protected nucleoside-
 polystyrene resin



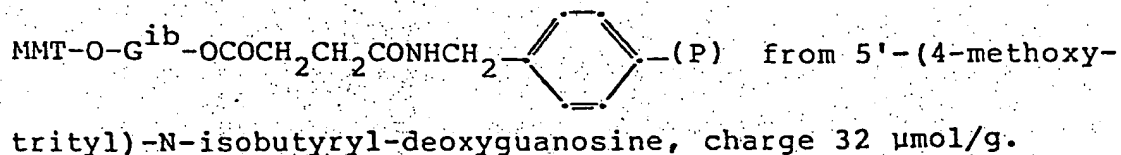
750 mg of succinic acid anhydride and 910 mg of 4-dimethylaminopyridine are added to 2.57 g (5 mmol) of 5'-(4-methoxytrityl)-thymidine (MMT-O-T-OH) in 20 ml of absolute pyridine and the whole is left at room temperature for 16 hours. After concentrating the pyridine solution, this is taken up in 200 ml of ethyl acetate, extracted by shaking twice, with 200 ml of 0.1M phosphate buffer each time with the addition of 10 ml of saturated sodium chloride solution, washed again with saturated sodium chloride solution, dried, concentrated and hexane is added dropwise. The precipitated product is separated off and triturated twice with ether, then dissolved in 300 ml of ethyl acetate and extracted by shaking at 0°C with 180 ml of 0.1M potassium bisulphate of pH 2.5. After washing twice with water, the ethyl acetate solution is dried with sodium sulphate, filtered, 0.5 ml of pyridine is added and the whole is concentrated and diluted by the dropwise addition of hexane. The precipitated succinic acid derivative is removed by filtration.

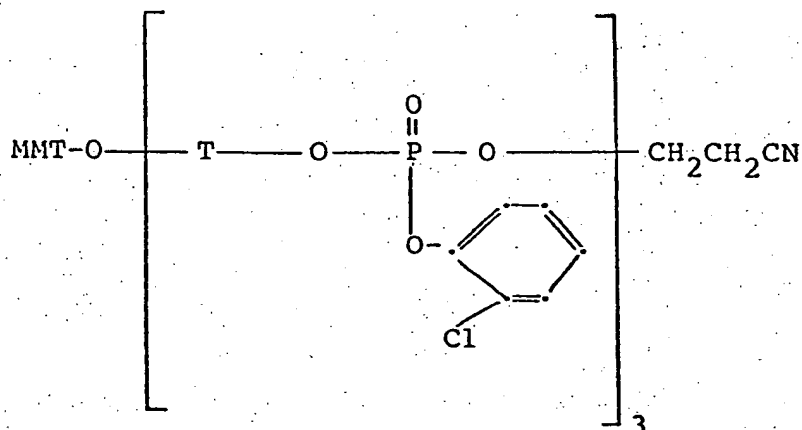
1.0 g of this compound are dissolved together with 190 mg of N-hydroxysuccinimide in 4 ml of ethyl acetate and 2 ml of dimethylformamide, and 370 mg of N,N'-dicyclohexylcarbodiimide are added at 0°C. After standing overnight in a refrigerator, the precipitated N,N'-dicyclohexyl urea is filtered off, the filtrate is diluted with ethyl acetate, extracted with cold 0.1M sodium bicarbonate and water, dried and concentrated to dryness by evaporation in vacuo. The residue is chromatographed with ethyl acetate on silica gel. TLC: R_f 0.45 in dichloromethane/methanol (9:1).

100 mg of this N-succinimidoylsuccinic acid ester are stirred for 20 hours with 1 g of aminomethylpolystyrene (amine content 110 µmol/g) in 2 ml of dichloromethane and 4 ml of dimethylformamide. The polymer resin is filtered off and extracted by washing with dimethylformamide, methanol, dichloromethane and methanol. After drying, the amino groups that have not reacted are acetylated by stirring the resin in 6 ml of pyridine with 1 ml of acetic anhydride and 100 mg of 4-dimethylaminopyridine for 30 minutes. The polymer resin is extracted by washing with dichloromethane, dimethylformamide, methanol and dichloromethane, and dried until a constant weight is reached. The spectroscopic methoxytrityl (MMT)-determination gives a charge of 57 µmol/g.

Example 2:

The following protected nucleoside/polystyrene resin is manufactured analogously to Example 1:



Example 3: Synthesis of the trinucleotidea) Synthesis of the dinucleotide:

7.73 g (15 mmol) of 5'-(4-methoxytrityl)-thymidine (MMT-O-T-OH) are twice concentrated by evaporation with absolute pyridine. The residue is dissolved in 20 ml of absolute tetrahydrofuran and added dropwise to 80 ml of a 0.2M solution of 2-chlorophenyl-di-(1-benzotriazolyl)-phosphate in tetrahydrofuran while stirring and with the exclusion of moisture and the reaction mixture is stirred for 1 hour at room temperature. The resulting solution of 2-chlorophenyl-1-benzotriazolyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate is divided into 3 portions.

α) Hydrolysis to triethylammonium-2-chlorophenyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate:

100 ml of 0.5M triethylammonium bicarbonate are added to a third of the above solution of 2-chlorophenyl-1-benzotriazolyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate while cooling. After 15 minutes extraction is carried out with dichloromethane. The dichloromethane solution is washed with water, concentrated, and petroleum ether is added dropwise thereto. The resulting precipitate is filtered off with suction, extracted by washing with ether/petroleum ether 1:1 and dried in vacuo. TLC: R_f 0.35 in dichloromethane/methanol/water (75:22:3).

β) Esterification to 2-cyanoethyl-2-chlorophenyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate and removal of the 4-methoxytrityl protecting group:

1.3 ml of 2-cyanoethanol and 2 ml of pyridine are added to a third of the solution of 2-chlorophenyl-1-benzotriazolyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate. The mixture is left to stand overnight at room temperature. The solvents are distilled in vacuo and the residue is dissolved in ethyl acetate and repeatedly extracted by shaking with 0.1M phosphate buffer of pH 7 and water. The organic phase is dried, concentrated and added dropwise to hexane. The precipitate is filtered off, dissolved in 50 ml of dichloromethane/methanol 7:3 and, at 0°C, a solution of 3.8 g of *p*-toluene-sulphonic acid monohydrate in 75 ml of dichloromethane/methanol 7:3 is added. After 2 hours, the reaction solution is diluted with dichloromethane and extracted by shaking with a cold sodium bicarbonate solution. The organic phase is concentrated and hexane is added. The precipitated 2-cyanoethyl-2-chlorophenyl-thymidine-3'-phosphate is chromatographed on silica gel with dichloromethane/-methanol 96:4. TLC: R_f of 0.45 in dichloromethane/methanol (9:1).

γ) Condensation to the 5'-(4-methoxytrityl)-3'-cyanoethyl-bis-thymidine dinucleotide:

2.2 g of 2-cyanoethyl-2-chlorophenyl-thymidine-3'-phosphate are dehydrated by twice concentrating by evaporation with absolute pyridine, and the product is dissolved in 20 ml of absolute tetrahydrofuran and added to the remaining third of the solution of 2-chlorophenyl-1-benzotriazolyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate. After 18 hours at room temperature 10 ml of water and 200 ml of ethyl acetate are added to the reaction solution while cooling with

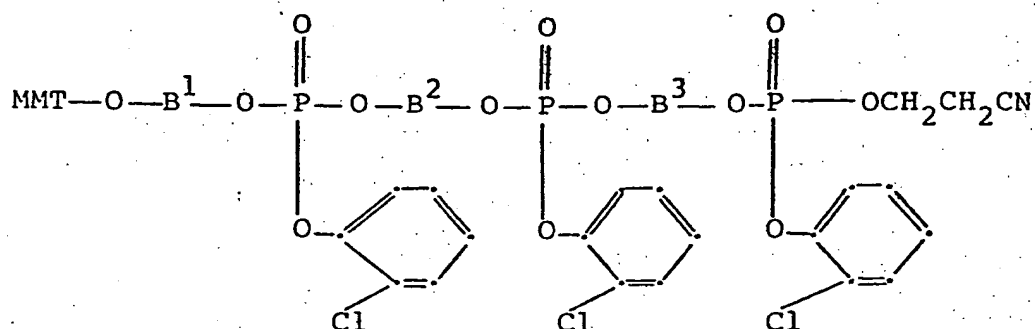
ice. The organic phase is washed repeatedly with sodium bicarbonate and water, dried over sodium sulphate and concentrated to a small volume. The dinucleotide, protected in the phosphate moiety and at the 5'- and 3'-end, is precipitated by adding dropwise to ether/hexane 1:1. TLC: R_f 0.48 in dichloromethane/methanol (9:1).

b) Synthesis of the trinucleotide:

1.17 g (1 mmol) of the above-described fully protected dinucleotide are dissolved in 30 ml of dichloromethane/methanol 7:3 and, while cooling with ice, a solution of 1.9 g of *p*-toluenesulphonic acid monohydrate in 20 ml of dichloromethane/methanol 7:3 is added. After 2 hours, ice-cold sodium bicarbonate solution is added and extraction is carried out with dichloromethane. The organic phase is dried, concentrated and added dropwise to hexane. The precipitated crude dinucleotide with a free 5'-hydroxy group is chromatographed on silica gel with a gradient of 2-8% methanol in dichloromethane. TLC: R_f 0.33 in dichloromethane/methanol (9:1).

850 mg of this 5'-hydroxy-dinucleotide and 1.06 g of triethylammonium-2-chlorophenyl-5'-(4-methoxytrityl)-thymidine-3'-phosphate [cf. paragraph a)] are twice concentrated by evaporation with pyridine, then dissolved in 10 ml of absolute pyridine and 560 mg of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) are added. After 2 hours, 2 ml of ice-cold water are added and, after a further hour, extraction is carried out with dichloromethane. The organic phase is washed with saturated sodium bicarbonate solution and water, dried, concentrated and ether is added. The precipitated trinucleotide is purified by chromatography on silica gel. R_f 0.45 in dichloromethane/methanol (9:1).

Example 4: Analogously to Example 3, the following protected trinucleotides of the general formula



abbreviated to $\text{B}^1\text{B}^2\text{B}^3$, are manufactured. The following abbreviations are used for the nucleosides B^1 , B^2 , B^3 :

A = N-benzoyl-deoxyadenosine

C = N-benzoyl-deoxycytidine

G = N-isobutyryl-deoxyguanosine

T = thymidine

Compound	$R_f^{a)}$	Compound	$R_f^{a)}$
TTT	0.45	ATG	0.48
TTC	0.55	ACT	0.53
TCT	0.46	ACC	0.48
TAC	0.56	ATT	0.55
TGC	0.44	ACA	0.53
TAG	0.60	AAC	0.46
TGT	0.42	AAA	0.51
TGA	0.44	AGT	0.45
CTT	0.53	AGG	0.38
CTG	0.46	GTT	0.45
CCT	0.45	GTC	0.44
CCC	0.59	GTG	0.35
CCG	0.47	GCA	0.49
CCA	0.51	GCG	0.48
CAT	0.55	GAT	0.44
CAC	0.51	GAC	0.48

Compound	R _f ^{a)}	Compound	R _f ^{a)}
CGC	0.46	GAG	0.48
CGA	0.38	GAA	0.50
CAG	0.44	GGC	0.42
CGG	0.38	GGT	0.46
CGT	0.49	GGG	0.35
		GGA	0.44

a) Thin layer chromatogram on silica gel in dichloromethane/methanol 9:1

Example 5: Synthesis of the DNA fragment of a length of 67 bases from base No. 96 to base No. 162 of the DNA strand 96/97)

a) Removal of the 2-cyanoethyl protecting group from the trinucleotides:

10 µmol of the trinucleotides from Example 3 or 4 are dissolved, with the exclusion of moisture, in 60 µl of pyridine/acetonitrile/triethylamine 1:1:1. After 1 hour at room temperature, 0.7 ml of peroxide-free ether is added dropwise and the precipitate is removed by centrifuging. The crude triethylammonium salt is dissolved in 50 µl of pyridine and again precipitated with 0.5 ml of ether, centrifuged off, and dried in a high vacuum for 15 hours.

b) Coupling the partially protected trinucleotides with the oligonucleotide chain bonded to polystyrene resin:

All operations are carried out with the exclusion of moisture in a reaction vessel of 220 µl capacity with microprocessor-controlled addition of solvent and reagent. 13 mg (0.74 µmol) of the thymidine/poly-

styrene resin (Example 1) are placed in the reaction vessel and subjected to the following operations:

1. Methylene chloride, 2 ml/min., 4 min.
2. Methylene chloride/isopropanol (85:15), 2 ml/min., 2 min.
3. Zinc bromide 1M and 1,2,4-triazole 0.02M in methylene chloride/isopropanol (85:15), 2 ml/min., 2-3.5 min.
4. Methylene chloride/isopropanol (85:15), 2 ml/min., 4 min.
5. Triethylammonium acetate 0.5M in DMF, 2 ml/min., 5 min.
6. Molecular sieve-dried pyridine, 2 ml/min., 3 min.
7. Tetrahydrofuran (peroxide-free, molecular sieve-dried), 2 ml/min., 3 min.
8. Nitrogen stream, 10 min.
9. Injection of 10 μ mol of trinucleotide GTC (tri-methylammonium salt from paragraph a)) and 8.9 mg (30 μ mol) of 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) dissolved in 150 μ l of pyridine.
10. 45°C, 20 min.
11. Pyridine, 2 ml/min., 4 min.
12. Acetic anhydride 5% and 4-dimethylaminopyridine 2.5% in pyridine, 2 ml/min., 4 min.
13. Pyridine, 2 ml/min., 4 min.
14. Pyridine/isopropanol (1:1), 2 ml/min., 3 min.

All 14 operations are repeated 21 times, but in the 9th operation, instead of GTC the following trinucleotides, respectively, are used in the form of their triethylammonium salts (paragraph a)) in the sequence indicated: GCA, ACC, GAA, CCC, TAC, AGG, TGA, CGC, TAC, CGT, GTG, CCA, AAA, AAA, TGA, CGG, TGA, TTC, GGG, CCT, CAT. The mean coupling yield is 97%. The end product has the following structure:

MMT-CATCCTGGGTTCTGACGGTGAAAAAAACCAGTGC GTTACCGGTGAAGGTACCCCGAAACCGCAG

TCT-polystyrene

c) Removal of the DNA fragment from the carrier and removal of the protecting groups:

40.0 mg (approximately 0.60 μmol) of DNA synthesis resin 96/67 are maintained at 50°C for 3 hours and at room temperature for 12 hours with 66 mg (0.40 mmol) of *o*-nitrobenzaloxime and 50 μl (0.40 mmol) of 1,1,3,3-tetramethylguanidine in 400 μl of 95% pyridine. After blowing off the pyridine with nitrogen, 1.6 ml of aqueous ammonia (33%) are added to the residue and the whole is maintained for 24 hours at 50°C in a closed vessel.

The liquid phase separated off is freed of ammonia in vacuo and washed 3 times with 3 ml of peroxide-free diethyl ether each time. After removing the lower molecular constituents on a Biogel P6 column (100-200 mesh, 3x66 cm, 0.01 molar trimethylammonium bicarbonate pH 7.5, 1.5 ml/min.), 285 ODs (260 nm) of DNA are isolated.

A total of 60 ODs are separated on an HPLC column (PRP-1/Hamilton, 250 x 4.6 mm). Gradient (solution A: 0.05M triethylammonium acetate pH 7.0; solution B: solution A:acetonitrile 1:1): 30 % B in A 60 % B in A in 20 min. at 50°C and 2 ml/min. The lipophilic main peak (retention time approximately 14 min.) is collected, concentrated over a DE52 cellulose (Whatman) column, eluted and precipitated with ethanol. To remove the 4-methoxytrityl protecting group, the precipitate is dissolved in 50 μl of acetic acid/H₂O (4:1) and maintained at room temperature for 45 minutes. The reaction product is lyophilised, precipitated with ethanol and, for purification, electrophoretically separated on an 8% polyacrylamide gel (7M urea). The

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band corresponding to the desired DNA size is cut out and the product is electroeluted, concentrated over DE52 cellulose and the DNA 96/97 of the following structure

5'-CATCCTGGGTCTCTGACGGTGAAAAAACCAGTCCGTTACCGGTGAAGGTACCCGAAACCG
CAGTCT-3'

is precipitated with ethanol.

Example 6: The following DNA fragments (5'-3') are produced analogously to Example 5:

1/58

CTGGAATTCATCGTTGTTTACACCGACTGCACCGAATCTGGTCAGAACCTGTGCCGTG

46/64 complementary

CAGAACCCAGGATGCATTGTGTTACCCGTACCCGCAAACGTTAGAACCCTTCGCACAGGCACAGGT

154/64 complementary

CAGGATCCTACTGCAGGTATTCTTCCGGGATTCTTCGAAGTCACCGTCGTTGTGAGACTGCCG

Example 7: Phosphorylation of the fragments 1/58,
46/64 complementary, 96/67 and 154/65
complementary

The phosphorylation and the radioactive labelling at the 5'-ends is effected with [γ -³²P]ATP and T₄ polynucleotide kinase (Boehringer) as described in Molecular Cloning, A laboratory Manual (ed. T. Maniatis et al.), Cold Spring Harbor Lab., 1982, p. 125

Example 8: Polymerisation to duplex II (fragment F₂ of the desulphatohirudin HV1 gene)

50 pmol in each case of kinased fragment 96/67 and kinased fragment 154/64 are dissolved in 24 µl of water, the solution is heated at 90°C for 3 minutes and cooled to 12°C within a period of 5 minutes.

After the addition of 4 µl of endo-R buffer (0.1 molar tris·HCl pH 7.5, 66 mM MgCl₂, 66 mM β-mercaptoethanol, 0.6M NaCl), 10 µl of deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, TTP, each 2x10⁻³ molar, adjusted with NH₃ to pH 7.0) and 2 µl (10 units) of DNA polymerase I, Klenow fragment (Boehringer) incubation is carried out for 30 minutes at 12°C. The reaction is stopped by heating at 90°C for 3 minutes and the mixture is stored at -80°C until required for further processing.

In an analogous manner the kinased fragments 1/58 and 46/64 are reacted to form duplex I (fragment F₁ of the desulphatohirudin gene).

Duplexes I and II have the following structures:

Duplex I

CTGGAATTCATGGTTGTTTACACCGACTGCACCSAATCTGGTCAGAACCTGTGCCTGTGCGAAGGTTG
GACCTTAAGTACCAACAAATGTGGCTGACGTGGCTTAGACCAGTCTTGGACACGGACACGCTTCCAAG

TAACGTTTGGCGTCAGGGTAACAAATGCATCCTGGGTTCTG
ATTGCAACGCCAGTCCCATTTGTTTACGTAGGACCCAAGAC

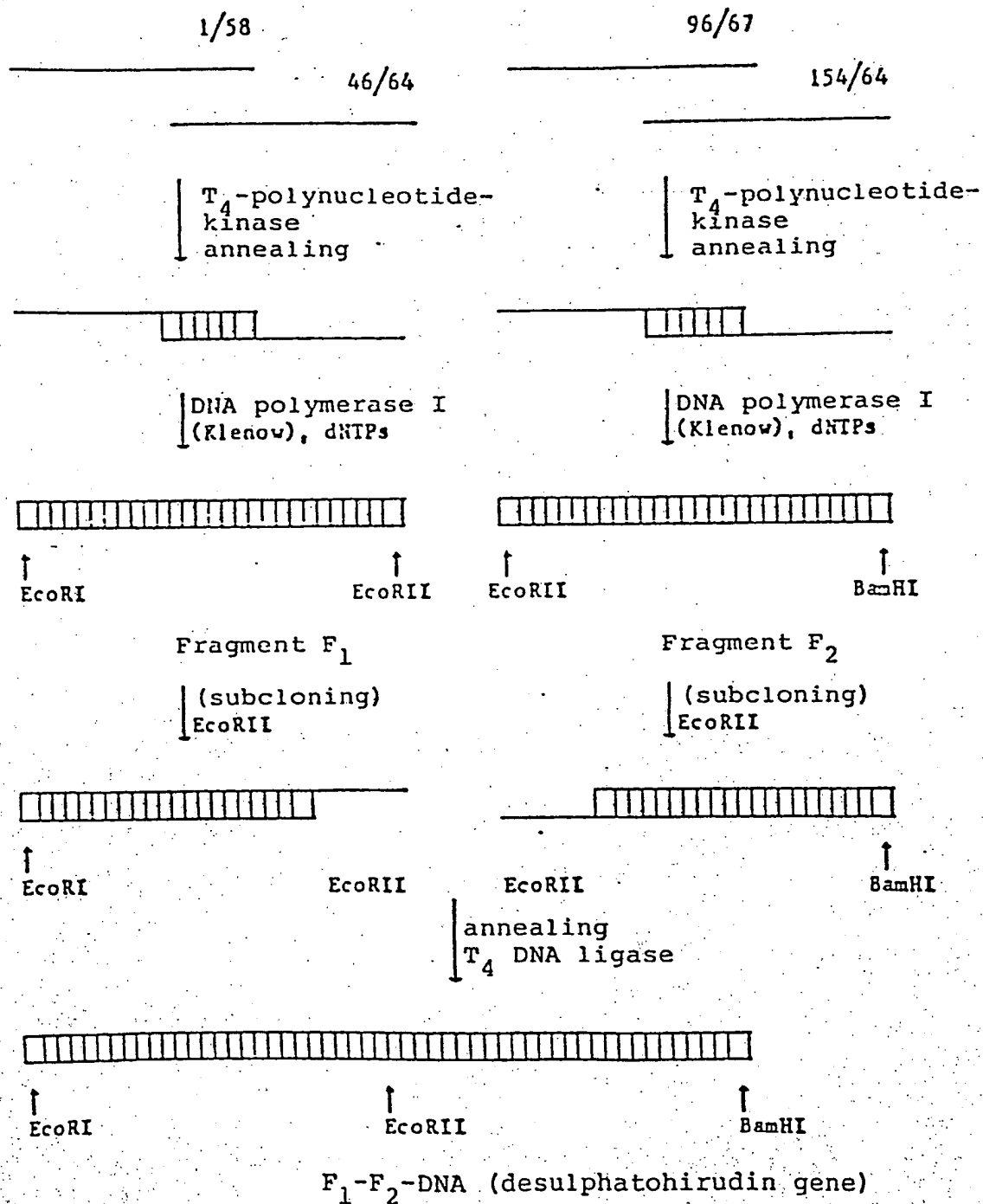
Duplex II

CATCCTGGGTTCTGACGGTGAAAAAAACCAGTGCGTTACCGGTGAAGGTACCCCGAAACCCGAGTCTG
GTAGGACCCAAGACTGCCACTTTTTTTGGTCACGCAATGGCCACTTCCATGGGGCTTTGGCGTCAGAG

ACAACGACGGTGACTTCGAAGAAATCCCGGAAGAATACCTGCAGTAGGATCCTG
TGTTGCTGCCACTGAAGCTTCTTTAGGGCTTCTTATGGACGTCATCCTAGGAC

The manufacture of the fragments F₁ and F₂ of the desulphatohirudin gene is illustrated in the following scheme 1:

Scheme: 1 Manufacture of the fragments F_1 and F_2 of the desulphatohirudin gene HV1 and of F_1-F_2 DNA



Example 9: Polymerisation and ligation of the fragments 1/58, kinased 46/64, kinased 96/67, and 154/64; manufacture of the desulphatohirudin HVL gene

In each case 50 pmol of fragment 1/58, kinased 46/64, kinased 96/67 and 154/64 are dissolved in 48 μ l of water, the solution is heated for 3 minutes at 90°C and cooled within 5 minutes to 12°C. After the addition of 8 μ l of Endo-R buffer (cf. Example 8), 20 μ l of deoxynucleoside triphosphate mixture (dATP, dCTP, dGTF, TTP, in each case 0.002 molar, adjusted to pH 7.0 with NH_3) and 2 μ l (10 units) of DNA polymerase I, Klenow fragment (Boehringer) incubation is carried out for 30 minutes at 12°C. The reaction is stopped by heating from 3 minutes at 90°C and the DNA is isolated, after extraction with phenol/chloroform, by precipitation with ethanol. The resulting DNA mixture is dissolved in 100 μ l of ligase/buffer (66 mM tris·HCl pH 7.5, 6.6 mM MgCl_2 , 10 mM dithiothreitol, 5 mM ATP), 50 units (2 μ l) of T_4 DNA ligase (Biolabs) are added and the whole is incubated for 20 hours at 20°C. The reaction is stopped by heating for 5 minutes at 70°C and the DNA, after extraction with phenol/chloroform, is isolated by precipitation with ethanol. After separating the mixture on an 8% polyacrylamide gel (denaturing) by electrophoresis, the ligation product with 217 base pairs is electroeluted, concentrated on a DE52 cellulose column and, after elution, isolated by precipitation with ethanol.

The desulphatohirudin gene has the following structure.

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CTGGAATTCATGGTTGTTTACACCGACTGCACCGAATCTGGTCAGAACCTGTGCCTGTGCGAAGGT
 GACCTTAAGTACCAACAAATGTGGCTGACGTGGCTTAGACCAGTCTTGGACACGGACACGCTTCCA

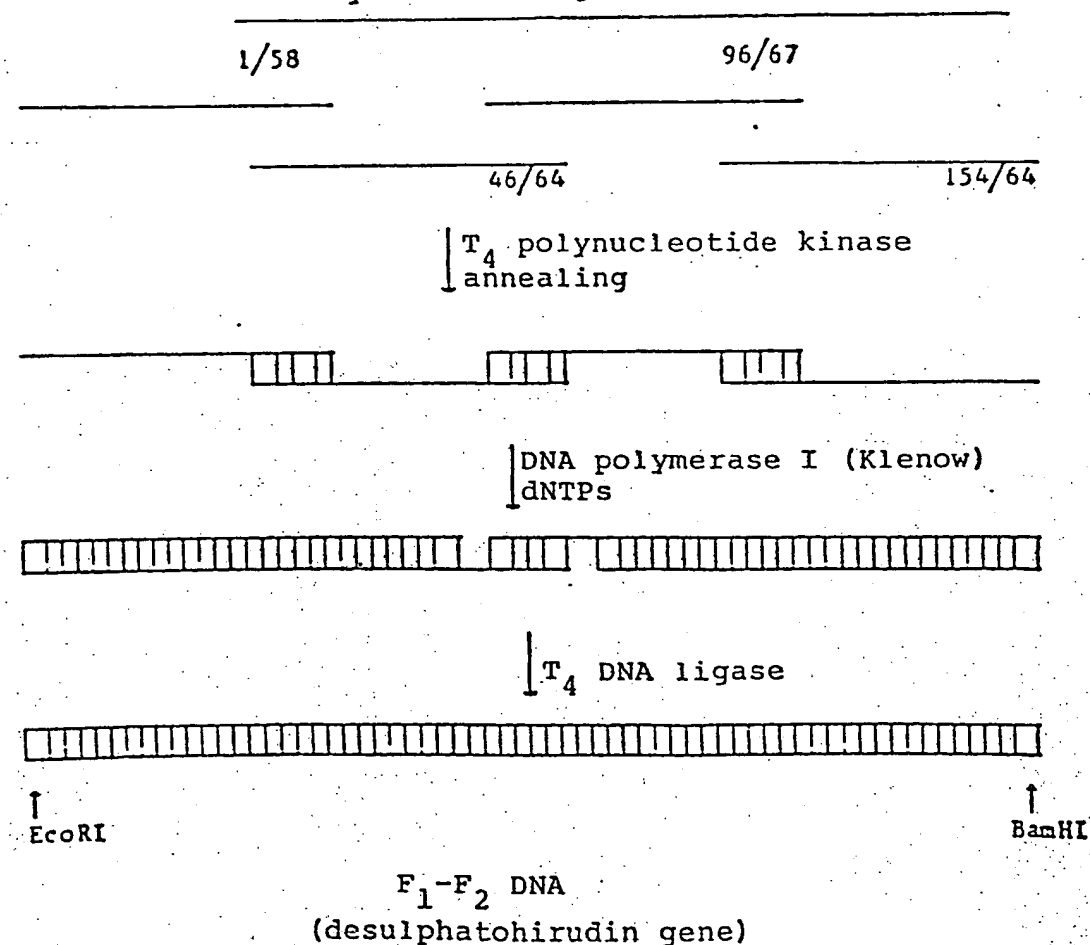
TCTAACGTTTCCGGTCAGGGTAACAAATGCATCCTGGGTTCTGACGGTGAAAAAACCAGTGCCTT
 AGATTGCAAACGCCAGTCCCATTTGTTTACGTAGGACCCAAGACTGCCACTTTTTTTGGTCACGCAA

ACCGGTGAAGGTACCCCGAAACCGCAGTCTCACAACGACGGTGACTTCCGAAGAAATCCCGGAAGAA
 TGGCCACTTCCATGGGGCTTTGGCGTCAGAGTGTGCTGCCACTGAAGCTTCTTTAGGGCCTTCTT

TACCTGCAGTAGGATCCTG
 ATGGACGTCATCCTAGGAC

The manufacture of the desulphatohirudin gene from the four fragments is illustrated in the following scheme 2:

Scheme 2: Manufacture of the hirudin gene from the four synthetic fragments



Example 10: Construction of the plasmid pML 300 containing the F₁-DNA of the desulphatohirudin HV1 gene (Fig. 1)

a) Manufacture of the linearised vector pBR322/EcoRI/PvuII.

5 µg of pBR322 plasmid DNA are digested with 5 units of PvuII restriction endonuclease (Biolabs) in 200 µl of a solution of 100 µg/ml of gelatin for 1 hour at 37°C. Subsequently this solution is adjusted to 100 mM tris-HCl (pH 7.5), 50 mM NaCl and the DNA is digested with 30 units of EcoRI restriction endonuclease (Biolabs) for 2 hours at 37°C. The solution is then adjusted to 50 mM tris-HCl pH 8 and, at a DNA concentration of 10 µg/µl, incubated with 2 units of intestinal alkaline calf phosphatase (Boehringer) for 30 mins at 37°C. The enzyme is inactivated by heating the solution for 60 minutes at 65°C. The solution is then standardised with TNE, extracted with 1 volume of phenol and chloroform, and the digested DNA is precipitated with 2 volumes of alcohol at -20°C overnight.

The vector (pBR322/EcoRI/PvuII, 2297 base pairs) excised from the pBR322 DNA is separated from the small DNA fragment (2067 base pairs) by gel electrophoresis on 1 % low-melting agarose (Biorad) in tris-acetate-EDTA buffer pH 8. After staining the DNA in the agarose gel with EtBr, the area of the gel that contains the DNA band of the pBR322/EcoRI/PvuII vector (= 2297 base pairs) is cut out of the gel and liquefied for 10 minutes at 65°C. 20 volumes of TNE are added to this DNA solution, the DNA is purified in accordance with Mueller et al. [J. Mol. Biol. 124, 343, (1978)] by DE-52 chromatography, extracted with phenol/chloroform and the DNA is precipitated with alcohol at -20°C

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overnight. The DNA precipitate is dissolved in 50 μ l of 0.01M tris·HCl (pH 8), 0.1 mM EDTA, and stored at -20°C until required for use. 1.4 μ g (= 3.2 pmol of ends) of DNA are obtained.

b) Manufacture of F_1 -DNA/EcoRI

24 ng (= 1.3 pmol of ends) of the chemically synthesised F_1 -DNA (see Example 8) are digested with 5 units of EcoRI restriction endonuclease (Biolabs) in 50 μ l of 100 mM tris·HCl (pH 7.5), 50 mM NaCl and 100 μ g/ml gelatin for 30 minutes at 37°C . Subsequently, 0.06 μ g (= 0.14 pmol of ends) of the linearised vector pBR322/EcoRI/PvuII (Example 10a) are added to the solution. The enzyme is then, by heating at 65°C , inactivated after 10 minutes, and the solution is standardised with TNE and extracted with phenol/chloroform. The DNA is precipitated with alcohol. The precipitated DNA is stored under alcohol at -20°C until further processing.

c) Ligation of pBR322/EcoRI/PvuII vector DNA with F_1 -DNA/EcoRI and construction of plasmid pML300

The DNA precipitate obtained in Example 10b), which contains the two mentioned DNA fragments, is dissolved in 20 μ l of a solution of 50 mM tris·HCl (pH 7.8), 10 mM MgCl_2 , 10 mM DTT, 0.5 mM ATP, and 100 μ g/1 gelatin and treated with 25 units/ μ l T_4 DNA ligase (Biolabs) at 15°C for 3 hours. In this manner the recombinant plasmid pML300, which contains the F_1 -DNA, is obtained in the solution.

d) Transformation of E. coli HN101 with plasmid pML300

The E. coli HB101 cells pretreated with calcium that are required for the transformation are produced as described by Mandel et al.

[J. Mol. Biol. 53, 159 (1970)].

The solution obtained in c), which contains the recombinant plasmid pML300, is heated at 65°C for 10 minutes in order to inactivate the T₄ DNA ligase and is then cooled to 37°C. 10 µl of this reaction mixture are added to 150 µl of calcium-treated E. coli HB101 cells in 10 mM MgCl₂ and 10 mM tris·HCl (pH 7.5) in a total volume of 200 µl.

Subsequently, this mixture is cooled in ice for 30 minutes, heated for 2 minutes at 42°C and then left to stand for 50 minutes in 1 ml of L-medium (cf. Example 18) at 37°C. The mixture is then spread out in aliquots of 0.2 ml on 5 agar plates (McConkey Agar, Difco), which contain 60 µg/ml of ampicillin (Serva). The agar plates are then maintained at 37°C for 16-18 hours. 484 ampicillin-resistant colonies of the transformed E. coli HB101 are obtained.

e) Screening the colonies that contain F₁-DNA

470 transformed colonies (Example 10d) are pressed off onto nitrocellulose filter B85 (Schleicher and Schull). In accordance with M. Grunstein and D.S. Hogness [Proc. Natl. Acad. Sci. USA 72, 3961 (1979)] the colonies are lysed and their denatured DNA is fixed on the filter. Subsequently prehybridisation of the filters is carried out in 20 ml (per filter) of 4xSET [= solution of 30 mM tris·HCl (pH 8), 150 mM NaCl, 1 mM EDTA], 0.1 % (w/v) Ficoll 400 (Pharmacia), 0.5 % SDS, 50 µg/ml denatured calf thymus DNA for 4 hours at 64°C. Subsequently the nitrocellulose filters are treated in 20 ml (per filter) of 5xSET (w/v) Ficoll

400, 0.2 % SDS and 50 µg/ml denatured calf thymus DNA for 16 hours at 64°C with the ³²P radioactively labelled probe (approximately 10³-10⁴ Cerencov cpm per filter). The oligonucleotide 46/64 complementary (cf. Example 6) is used as probe.

Subsequently, the filters are washed twice in 2xSET, 0.2 % SDS at room temperature, then twice in 2xSET, 0.5 % SDS at 60°C (first for 30 minutes, then for 60 minutes). The filters are then dried between 3 MM paper (Whatman) and placed at -80°C on an X-ray film (Fuji) with an intensifying screen (Ilford) for 1-2 days.

The resulting autoradiogram shows 71 positive colonies (clones) which can be used for further processing, one of which received the designation pML300.

Example 11: Construction of plasmid pML305, which contains the F₂-DNA of the desulphatohirudin HV1 gene (Fig. 2)

a) Manufacture of linearised vector pBR322/BamHI/NruI

5 µg of pBR322 plasmid DNA are digested with 30 units of BamHI restriction endonuclease for 30 minutes at 37°C in a solution of 100 mM NaCl, 6 mM tris·HCl (pH 7.9), 6 mM MgCl₂ and 100 µg/ml gelatin. 15 units of PvuII restriction endonuclease are then added to the solution and the solution is digested for 2 hours at 37°C.

The reaction mixture is heated at 70°C for 10 minutes in order to inactivate the enzyme. Subsequently the two DNA fragments are separated from each other by gel electrophoresis on 1 % low-melting agarose in tris-acetate-EDTA buffer, pH 8. (see Example 10a).

The DNA band of the pBR322 BamHI/PvuII vector (= 2672 base pairs) is cut out, liquefied, and purified in accordance with Mueller et al.(supra) by DE-52 chromatography. 0.75 μ g (= 1.5 pmol of ends) of DNA are obtained.

b) Manufacture of F₂-DNA/BamHI

25 μ g (= 1.3 pmol of ends) of chemically synthesised F₂-DNA (Example 8) are digested with 16 units of BamHI restriction endonuclease (Biolabs) in 20 μ l of 150 mM NaCl, 6 mM tris·HCl (pH 7.9), 6 mM MgCl₂ and 100 μ g/ml gelatin for 30 minutes at 37°C. 60 ng (= 96 nmol of ends) of the linearised vector pBR322/BamHI/PvuII (Example 11a) are then added to the solution, the whole solution is standardised with TNE and extracted with phenol/chloroform, and the DNA is precipitated with 2 volumes of alcohol. The precipitated DNA is stored under alcohol at -20°C until required for further processing.

c) Ligation of pBR322/BamHI/PvuII vector DNA with F₂-DNA/BamHI and construction of plasmid pML305

The DNA precipitate obtained in Example 11b), which contains the two mentioned DNA fragments, is dissolved in 20 μ l of a solution of 50 mM tris·HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP, and 100 μ g/ml gelatine and treated with 15 units/ μ l T₄ DNA ligase (Biolabs) at 15°C for 3 hours. In this manner the recombinant plasmid pML305, which contains F₂-DNA, is obtained in the solution.

d) Transformation of E. coli HB101 with plasmid pML305

The transformation of the calcium-treated E. coli HB101 cells is carried out as described in Example 10d). 10 μ l of the reaction mixture obtained in Example 11c) are used. 313 ampicillin-resistant colonies are obtained.

e) Screening the colonies that contain F₂-DNA

65 transformed colonies (Example 11d) are examined for F₂-DNA in the manner described in Example 10e). The oligonucleotide 154/64 complementary (cf. Example 6) is used as radioactive probe. 2 positive colonies are obtained in the autoradiogram, one of which is designated pML305.

Example 12: Characterisation of clones pML300 and pML305

The DNAs of recombinant plasmids pML300 and pML305 are isolated in accordance with Ish-Horowitz [Molecular Cloning, A laboratory Manual (ed. T. Maniatis et al.), Cold Spring Harbor Lab., 1982, p. 368]. The nucleotide sequences of the F₁-DNA and F₂-DNA inserts are ascertained in accordance with Maxam and Gilbert [Proc. Natl. Acad. Sci. USA 74, 560 (1977); see also Meth. Enzym. 65, 499 (1980)].

For this purpose, in each case 10 μ g of plasmid DNA of pML300 are cleaved with EcoRI restriction endonuclease and 10 μ g of plasmid DNA of pML305 are cleaved with BamHI restriction endonuclease and the linearised DNAs are isolated by gel elution from agarose gel [cf. Example 10a)]. Subsequently the isolated DNAs are digested with alkaline phosphatase and chromatographed over DE-52 (cf. Example 11a). The DNAs are then radioactively labelled at the 5'-end with [γ -³²P]ATP (specific activity 5000 Ci/mmol, Amersham) and T₄ polynucleotide kinase (P-L-Biochemicals).

The radioactively labelled DNAs are then cleaved with a second restriction endonuclease (EcoRII). The resulting DNA fragments are isolated by gel elution from agarose. In the case of pML300 the nucleotide sequence of F₁-DNA is then determined from the EcoRII-EcoRI* fragment (approximately 109 base pairs) and in the case of pML300 that of F₂-DNA is determined in the EcoRII-BamHI* fragment (approximately 122 base pairs).

(* indicates the DNA end that is radioactively labelled).

The nucleotide sequences that are determined for F₁-DNA and F₂-DNA are identical to those illustrated in Example 8.

Example 13: Construction of expression plasmid pML310

- a) Construction of linearised vector pHRi148/EcoRI/BamHI, which contains the trp promotor-operator (Fig. 3 and Fig. 4)

A. Construction of plasmid p159

10 µg of plasmid pBRH_{trp} [DE-OS 3 111 405] are cleaved with 50 units of EcoRI (Biolabs) for 60 minutes at 37°C and the digestion mixture, after phenol extraction, is fractionated on a saccharose density gradient (5-23%) in 50 mM tris·HCl (pH 8.0), 1 mM EDTA in a TST41 (Kontron AG) rotor. The centrifugation lasts for 14 hours at 40,000 revs/min and 15°C. 0.3 ml fractions are collected with an ISCO gradient collector at 1 ml/min. The fractions that contain the smaller fragment are combined, the solution is standardised with TNE and precipitation is effected with 2 volumes of ethanol at -20°C. After centrifugation in an Eppendorf centrifuge, the DNA is dissolved in 100 µl of

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10 mM tris·HCl pH 7.5, 0.5 mM EDTA. 5 µg of this DNA fragment are cleaved with 5 units of Bg1II (Biolabs) for 60 minutes at 37°C. The reaction mixture is extracted with phenol and chloroform and the DNA is incubated with 2 volumes of ethanol at -80°C for 10 minutes; the DNA is collected by centrifugation and dissolved again in 50 µl of 50 mM tris·HCl (pH 8.0). 2 µl of this solution are removed (0.2 µg of DNA) and incubated at a DNA concentration of 10 ng/µl in 50 mM tris·HCl (pH 8.0) with 1 unit of intestinal alkaline calf phosphatase (Boehringer) for 30 minutes at 37°C. The enzyme is inactivated by heating the solution for 60 minutes at 65°C. 0.04 µg of DNA is removed and incubated 5'-terminally with 10 µCi [γ -³²P]-ATP (5000 Ci/mmol, Amersham) and 5 units of T₄ polynucleotide kinase (P-L Biochemicals) in 20 µl of reaction volume in 50 mM tris·HCl (pH 9.5), 10 mM MgCl₂, and 5 mM DTT, for 30 minutes at 37°C. The radioactive sample is mixed with the non-labelled sample (see above) and the DNA fragments are fractionated by a 5-23 % saccharose density gradient in 50 mM tris·HCl (pH 8.0), 1 mM EDTA in a TST60 rotor. The centrifugation is carried out for 5 hours at 60,000 revs/min and 15°C. 0.2 ml fractions are collected. The radioactivity of each fraction is determined by measuring the Cerencov radiation and the fragments are thereby identified. The desired fractions, which contain the small DNA fragment, are combined, the DNA is precipitated with 2 volumes of ethanol and, after centrifugation, is dissolved again in 20 µl of 10 mM tris·HCl pH 7.5, 0.5 mM EDTA.

The ³²P-labelled EcoRI-Bg1II DNA fragment is partially cleaved with 0.2 units of TaqI (Biolabs) in 50 µl volume for 10 minutes at 37°C. The reaction mixture is adjusted to 0.2% SDS, 10% glycerin, 10 mM

EDTA, 0.05% bromophenol-blue, and the DNA fragments are separated on a 6% polyacrylamide gel in tris-borate-EDTA [A.C. Peacock et al., Biochemistry 6, 1818 (1967)]. The Band containing the desired EcoRI-TaqI (the largest partial fragment) is identified on the autoradiogram. This fragment (L. see Fig. 3) is extracted from the gel and purified [W. Müller et al., *supra*] and dissolved in 10 μ l of 10 mM tris·HCl pH 7.5, 1 mM EDTA. in 10 μ l of 10 mM tris·HCl pH 7.5, 1 mM EDTA.

There is used as acceptor plasmid pBR322, which is cleaved with ClaI and EcoRI: 2 μ g of pBR322 are digested with 4 units of ClaI (Biolabs) in 20 μ l of reaction volume for 60 minutes at 37°C. The protein is extracted with phenol and the DNA is then precipitated with 2 volumes of ethanol at -80°C for 10 minutes. The DNA is collected by centrifugation and then digested with 10 units of EcoRI (Biolabs) for 30 minutes at 37°C in 20 μ l of reaction volume. Subsequently, 2 volumes of 0.1M tris·HCl (pH 8.7) are added to the solution and the whole is incubated with 1 unit of alkaline calf phosphatase (Boehringer) at 37°C for 30 minutes. The phosphatase is then inactivated by incubation at 65°C for 60 minutes. 100 ng of the acceptor plasmid are incubated with 5 μ l of fragment L-DNA in 15 μ l of reaction volume in 10 mM MgCl₂, 20 mM tris·HCl (pH 7.8), 10 mM DTT, 0.5 mM ATP with 30 units per μ l of reaction volume of T₄ DNA ligase (Biolabs) for 2 hours.

5 μ l of this solution are added to a mixture that contains 150 ml of E. coli HB101 cells treated with calcium chloride (*supra*) in 10 mM MgCl₂, 10 mM CaCl₂ and 10 mM tris·HCl (pH 7.5) in a total volume of 200 μ l. The mixture is cooled for 20 minutes in ice, heated for 1 minute at 42°C and incubated for 10 minutes at 20°C. 1 ml of tryptone medium [tryptone medium contains 10 g of bacto-tryptone (Difco); 1 g of yeast extract (Difco); 1 g of glucose; 8 g of NaCl and 294 mg of CaCl₂·2H₂O

in 1 litre of distilled water] is added and the mixture is incubated for 30 minutes at 37°C while agitating at 300 revs/min. The mixture is plated on two agar plates (McConkey Agar, Difco; 0.6 ml/plate), supplemented with 50 µg/ml of ampicillin (Sigma). The plates are incubated for from 12 to 17 hours at 37°C.

The plasmid DNA of 10 different colonies is isolated as follows:

The colonies are, as above, used for inoculation of 10 ml of tryptone medium supplemented by 50 µg/ml of ampicillin, in a 25 ml Erlenmeyer flask. The cultures are agitated for from 15 to 18 hours at 37°C and 300 revs/min. The cells are harvested by centrifuging (Sorval, HS-4 rotor, 10 minutes at 4000 revs/min., 4°C). Approximately 0.1 g of cells are obtained, and these are resuspended in 1 ml of 50 mM tris·HCl (pH 8.0). 0.25 ml of lysozyme solution [10 mg/ml in 50 mM tris·HCl (pH 8.0); lysozyme is marketed by Sigma] are added and, after incubation for 10 minutes at 0°C, 0.15 ml of 0.5 mM EDTA (pH 7.5) are added. After a further 10 minutes at 0°C, 60 µl of 2% Triton X-100 (Merck) are added. After 30 minutes at 0°C the sample is centrifuged for 30 minutes at 15,000 revs/min and 4°C in a Sorval SA-600 rotor. The supernatant is deproteinated with 1 vol. of phenol (saturated with TNE). The phases are separated by centrifuging (Sorval HB-4 rotor) for 10 minutes at 5000 revs/min and 4°C. The upper phase is extracted twice with 1 vol. of chloroform. Pancreatic RNase A (Sigma; 10 mg/ml in TNE preheated for 10 minutes at 85°C) is added until a final concentration of 25 µg/ml is reached and the mixture is incubated for 40 minutes at 37°C. The solution is then adjusted with 1M NaCl and 10% polyethylene glycol 6000 (Fluka, treated for 20 minutes at 120°C in an autoclave) and incubated for

2 hours at -10°C . The precipitate is collected in a Sorval HB-4 rotor (20 minutes at 10,000 revs/min., 0°C) and dissolved again in 100 μl of TNE. The DNA solution is extracted with 1 volume of phenol and the DNA is precipitated with 2 volumes of ethanol for 10 minutes at -80°C . The precipitate is collected by centrifuging in an Eppendorf centrifuge and the DNA is dissolved again in 20 μl of 10 mM tris-HCl (pH 7.5) and 0.5 mM EDTA. 8 to 10 μg of plasmid DNA are obtained from a 10 ml culture.

The plasmid DNAs are analysed after digestion with the following restriction enzymes:

In each case 0.5 μg of plasmid DNA is cleaved with HpaI (Biolabs) and with HpaI (Biolabs) and EcoRI (Biolabs) with ClaI (Biolabs) according to standard directions, in accordance with the instructions of the enzyme manufacturer. The DNAs are fractionated on a 1% agarose gel in 40 mM tris-acetate (pH 7.8), 1 mM EDTA and 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The desired plasmids contain an HpaI site and, after digesting 3 times, yield in addition to the large DNA fragment 2 smaller fragments which are larger than the small EcoRI-ClaI fragment of pBR322. One of these plasmids is designated p159 (see Fig. 3).

B. Construction of plasmid pHRi145

2 μg of p159 DNA are digested with 10 units of EcoRI (Biolabs) for 30 minutes at 37°C . The DNA is extracted with phenol, precipitated with ethanol and, after centrifugation, dissolved in 10 μl of 10 mM tris-HCl (pH 7.5), 0.5 mM EDTA. The DNA digested with EcoRI is furthermore treated with 5 units of DNA polymerase (Klenow fragment) (Boehringer) in 10 mM MgCl_2 , 10 mM β -mercaptoethanol, 50 mM NaCl, 0.1 mM dATP (P&L Biochemicals), 0.1 mM dTTP (P&L Biochemicals)

for 15 minutes at 12°C. The polymerase is then inactivated by incubation at 85°C for 5 minutes. The reaction mixture is diluted in 20 mM tris·HCl (pH 7.8), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP (Sigma) by a factor of 10 and incubated with 30 units of T₄ DNA ligase per µl of reaction mixture for 1 hour at 15°C.

50 ng of the DNA are transformed in E. coli (in the manner described above) and plated out on McConkey agar plates supplemented with 50 µg/ml ampicillin.

The plasmid DNAs of 10 different colonies are isolated in the manner described above. The plasmid DNAs are analysed by digesting with EcoRI. The desired plasmids are EcoRI-resistant. The analysis is carried out in the manner described above. One of the desired plasmids is designated HRi145 (Fig. 3).

C. Construction of plasmid pHRi148

2 µg of pHRi145-DNA are treated with 5 units of ClaI (Boehringer) for 60 minutes at 37°C, then deproteinated by means of phenol extraction. The DNA is precipitated with ethanol and then dissolved in 20 µl of 10 mM tris·HCl (pH 7.5), 0.5 mM EDTA. The projecting ends are, as described above, made up with DNA polymerase I (Klenow fragment), except that dATP and dTTP are replaced by dCTP (P&L Biochemicals) and dGTP (P&L Biochemicals). The polymerase is inactivated by incubation at 85°C for 5 minutes. 2 volumes of 0.1M tris·HCl (pH 8.7) are added to the reaction mixture which is incubated with 0.5 units of calf phosphatase (Boehringer) for 30 minutes at 37°C. The reaction mixture is deproteinated by extraction with phenol. The DNA is precipitated with ethanol and dissolved in 8 µl of 10 mM tris·HCl (pH 7.5), 0.5 mM EDTA.

A chemically synthesised DNA linker of the formula

5'-GAATTCCATGGTACCATGGAATTC-3'

is phosphorylated at the 5' end by incubating 8 pmol of the linker with 5 μ Ci [γ - 32 P]-ATP (5500 Ci \cdot mmol $^{-1}$ Amersham) in 8 μ l of reaction volume that contains 0.1 mM rATP (Sigma), 50 mM tris \cdot HCl (pH 9.5), 10 mM MgCl $_2$, 5 mM DTT and 2 units of T $_4$ polynucleotide kinase (P&L Biochemicals) for 30 minutes at 37°C. The reaction is stopped by freezing at -80°C.

The radioactively labelled linker is then treated with 1 μ g of ClaI and phosphatase and ligated with pHRi145-DNA (see above) in a 20 μ l reaction volume that contains 0.5 mM rATP (Sigma), 10 mM DTT (Calbiochem), 20 mM tris \cdot HCl (pH 7.8), 1 mM MgCl $_2$ and 800 units of T $_4$ DNA ligase (Biolabs). The incubation is carried out for 2 hours at 15°C. The ligase is inactivated by incubation at 85°C for 10 minutes. Subsequently, 2 volumes of water are added, the sodium chloride concentration is adjusted to 10 mM and 20 units of KpnI (Biolabs) are added over 30 minutes at 37°C. After extraction with phenol and chloroform, the mixture is fractionated through 0.9% low-melting agarose gel (Biorad) in 40 mM tris \cdot acetate (pH 7.8), 1 mM EDTA and 0.5 μ g/ml ethidium bromide. The band, visible by UV radiation, that demonstrates the same mobility as a marker DNA of the same size is cut out with a scalpel. The portion of gel is melted for 5 minutes at 65°C and then cooled to 37°C. A volume of approximately 20 μ l is obtained. 5 μ l of this solution are removed and incubated for 12 hours at 15°C with 400 units of T $_4$ ligase (Biolabs) in 10 μ l of reaction volume, which has been adjusted to 0.5 mM ATP, 10 mM DTT, 10 mM MgCl $_2$, 20 mM tris \cdot HCl (pH 7.8). 1/10 volume of a solution with 100 mM tris \cdot HCl (pH 7.5), 100 mM CaCl $_2$ and 100 mM MgCl $_2$ are added to

the ligase mixture (solidified at 15°C) and the whole is incubated at 65°C for 5 minutes. The solution is then used to transform calcium-treated E. coli HB101 cells in the manner described above. Plating out on McConkey Agar plates supplemented with 50 µg/ml ampicillin is carried out.

The plasmid DNAs of 10 different colonies are isolated in the manner described above, and the DNA is subjected to the following restriction enzyme analysis: In each case 0.5 µg of plasmid DNA is cleaved in succession with KpnI (Biolabs), NcoI (Biolabs) and EcoRI (Biolabs) in accordance with the instructions of the enzyme manufacturer. The cleavage products are fractionated on 1% agarose gels in 40 mM tris-acetate (pH 7.8), 1 mM EDTA, 0.5 µg/ml ethidium bromide. All plasmids exhibit one of these enzyme cleavage sites each, as desired. One is designated HRi148.

The plasmid HRi148 contains a tryptophan promoter-operator and a ribosomal binding site up to and including ATG. Hirudin and also other heterologous genes can be coupled directly by way of the EcoRI, NcoI, KpnI sites occurring once in the plasmid. Furthermore, this construction renders possible direct coupling and expressing of heterologous genes without the ATG necessary for the initiation of translation having to be present on the corresponding gene. This can easily be achieved by cleaving with NcoI and making up the projecting ends with DNA polymerase I, in the manner described, or by cleaving with KpnI and removing the projecting ends by nuclease S₁. The plasmid HRi148 is thus an expression plasmid with broad application.

D. Manufacture of the linearised vector pHRi148/
EcoRI/BamHI

5 µg of plasmid DNA of pHRi148 are digested with the restriction endonucleases EcoRI and BamHI. The excised vector pHRi148/EcoRI/BamHI is isolated by means of density gradient centrifugation.

b) Manufacture of F₁-DNA/EcoRI/EcoRII and
F₂-DNA/BamHI/EcoRII

I. Manufacture of F₁-DNA/EcoRI/EcoRII

5 µg of plasmid DNA of pML 300 are first digested with 10 units of EcoRI restriction endonuclease in 50 µl of a solution of 100 mM tris·HCl (pH 7.5), 50 mM NaCl and 100 µg/ml gelatine for 1 hour at 37°C. An aliquot (0.5 µg) of this linearised plasmid DNA/EcoRI is isolated by gel elution from an agarose gel (cf. Example 10a)) and radioactively labelled with [γ -³²P]ATP (cf. Example 12). The main amount of the plasmid DNA/EcoRI is then mixed with this radioactively labelled DNA, digested with EcoRII restriction endonuclease and the EcoRII-EcoRI* DNA fragment (109 base pairs) is separated by gel electrophoresis on 8% polyacrylamide. 200 µg of F₁-DNA/EcoRI*/EcoRII are isolated by gel elution.

II) Manufacture of F₂-DNA/BamHI/EcoRII

5 µg of plasmid DNA of pML 305 are cleaved with 10 units of BamHI restriction endonuclease. An aliquot (0.5 µg) of this linearised plasmid DNA/BamHI is isolated by gel elution from an agarose gel (cf. Example 10a)) and radioactively labelled with [γ -³²P] (cf. Example 12). The main amount of the plasmid

DNA/BamHI is then mixed with this radioactively labelled DNA, digested with EcoRII restriction endonuclease and the EcoRII-BamHI* DNA fragment (122 base pairs) is separated by gel electrophoresis on 8% polyacrylamide. 250 µg of the F₂-DNA/BamHI*/EcoRII are isolated.

c) Ligation of F₁-DNA with F₂-DNA and construction of the expression plasmid pML310

10 ng (= 473 nmol of ends) of F₁-DNA/EcoRI/EcoRII and 9 ng (= 495 nmol of ends) of F₂-DNA/BamHI/EcoRII are treated in a volume of 20 µl with T₄ DNA ligase in the manner already described in Example 10c). Subsequently, the mixture is extracted with phenol/chloroform and the DNA is precipitated with alcohol. The DNA precipitate is then dissolved as described in Example 10c) and digested with EcoRI and BamHI restriction endonuclease. The solution is then standardised with TNE and 30 ng (=50 nmol of ends) of the vector DNA pHRi148/EcoRI/BamHI (cf. Example 13aD) are added. Subsequently, the solution is again extracted with phenol/chloroform and the DNA is precipitated with alcohol. The precipitated DNA mixture is treated in the manner indicated in Example 10c) with T₄ DNA ligase (Biolabs). In this manner there is produced in the solution a recombinant plasmid that contains as insert F₁-F₂-DNA (desulphatohirudin gene).

d) Transformation of E. coli HB101 with plasmid pML310

The transformation of E. coli HB101 cells treated with calcium is carried out in the manner described in

Example 10d). 10 μ l of the reaction mixture obtained in Example 13c) are used. 420 ampicillin-resistant colonies are obtained.

e) Screening the colonies that contain F₁-F₂-DNA

18 transformed colonies (Example 13d) are examined for their F₁-F₂-DNA content in the manner described in Example 10e). A mixture of the oligodeoxynucleotides described in Examples 5 and 6 is used as radioactive probe. In the autoradiogram 12 positive colonies are obtained, five of which are designated pML310, pML311, pML312, pML313, pML314.

Example 14: Characterisation of the clone pML310

The characterisation of the F₁-F₂-DNA sequence in the recombinant plasmid pML310 is effected by sequencing the F₁-F₂-DNA according to Maxam and Gilbert (supra) in the manner described in Example 12. 10 μ g of plasmid DNA are examined. The nucleotide sequence of the F₁-F₂-DNA is identical to that described for the synthetic desulphatohirudin gene.

Example 15: Expression of desulphatohirudin HV1 in yeast

The chemically synthesized gene for hirudin is amplified as a 206 bp EcoRI-BamHI insert in plasmid pML310 (see example 14). The gene is excised from the plasmid and expressed in yeast under the control of the repressible PHO5 promoter. The construction includes the 541 bp PHO5 promoter sequence and the complete PHO5 signal sequence (51 nucleotides coding for 17 amino acids) as described in European Patent Application No. 143,081. This sequence is fused in frame to the mature coding sequence of hirudin which starts with the codon for valine as the NH₂-terminal amino acid

of natural mature hirudin. The hirudin gene is flanked at its 3' end by a DNA fragment providing the PHO5 transcription termination signals. The vector part of the expression plasmid contains yeast 2 μ sequences, the yeast LEU2 gene for selection in yeast and the ampicillin resistance gene and the E.coli origin of replication for selection and amplification in E.coli. The procedure is illustrated in Figures 5, 6 and 7.

Adjustment of the nucleotide sequence at the 5' end of the desulphatohirudin gene

The nucleotide sequence coding for desulphatohirudin starts with GTT which stands for the NH₂-terminal valine in the final gene product. For convenient subcloning and expression in E.coli the coding sequence has been extended at the 5' end by eight nucleotides including an EcoRI restriction site and an ATG initiation codon. For the exact in frame fusion of the hirudin coding sequence to the sequence coding for the PHO5 signal peptide these additional nucleotides have to be removed. This is achieved by converting the EcoRI restriction site to a flush end site, adding a synthetic oligonucleotide containing a HgaI recognition site in such a position that subsequent cleavage with HgaI occurs immediately upstream of the GTT codon.

Introduction of a HgaI restriction site in front of the desulphatohirudin gene (cf. Figure 5)

8 μ g of plasmid pML310 (see Example 13c) are digested to completion with restriction endonuclease EcoRI. The DNA (pML310/EcoRI) is extracted with phenol/chloroform and precipitated with ethanol. 5'overhanging ends are removed

by nuclease S_1 . 4 μ g of pML310/EcoRI DNA are digested in 100 μ l of 250 mM NaCl, 1 mM $ZnSO_4$, 30 mM sodium acetate pH 4.6 and 20 U/ml of nuclease S_1 (Sigma) for 45 min at 37°C.

The DNA is extracted with phenol/chloroform and precipitated by ethanol. The DNA (pML310/EcoRI/ S_1) is resuspended in 100 μ l of 50 mM Tris•HCl pH 8.0 and incubated with 2 units of calf intestine alkaline phosphatase (CIAP, Boehringer) for one hour at 37°C. The enzyme is inactivated at 65°C for 1.5 hours.

The NaCl concentration in the incubation mixture is adjusted to 150 mM. The dephosphorylated DNA (pML310/EcoRI/ S_1 /CIAP) is purified by adsorption to a DE52 (Whatman) ion exchange column in a low salt buffer (150 mM NaCl, 10 mM Tris•HCl pH 8.0, 1 mM EDTA) and then eluted with a high salt buffer solution (1.5 M NaCl, 10 mM Tris•HCl pH 8.0, 1 mM EDTA). The DNA is precipitated with ethanol and resuspended in H_2O at a concentration of 0.8 mg/ml.

An oligonucleotide of the formula



is synthesized by the phosphotriester method [Itakura et al., J. Am. Chem. Soc. 103, 706 (1981)]. The sequence of the oligonucleotide is self-complementary containing the recognition site -GACGC- for restriction endonuclease HgaI.

Annealing of two single strands leads to a double-stranded DNA linker of 12 base pairs.

1.2 μg of the synthetic single-stranded oligodeoxynucleotide are phosphorylated in 10 μl of 60 mM Tris $\cdot\text{HCl}$ pH 7.5, 10 mM MgCl_2 , 5 mM DTT, 30 μCi of [γ - ^{32}P] ATP (3000 Ci $\cdot\text{mmol}^{-1}$, Amersham) and 6 units of T_4 polynucleotide kinase (Boehringer) for 30 min at 37°C, followed by a 15 min chase at 37°C in the presence of 0.5 mM ATP. The mixture is further incubated for 10 min at 75°C to inactivate the enzyme and is then allowed to cool to room temperature for annealing.

0.6 μg (170 pmoles) of the ^{32}P -labelled linker DNA are mixed with 2.4 μg (1.75 pmol ends) of pML310/EcoRI/ S_1 /CIAP and ligated in 20 μl of 60 mM Tris $\cdot\text{HCl}$ pH 7.5, 10 mM MgCl_2 , 5 mM DTT, 3.5 mM ATP, 800 units of T_4 DNA ligase (Biolabs) for 20 hours at 15°C. The ligase is inactivated at 85°C for 10 min and the excess of linker molecules is removed by precipitation of the DNA in the presence of 10 mM EDTA pH 7.5, 300 mM sodium acetate pH 6.0 and 0.54 volumes of isopropanol. After 30 min at room temperature the DNA is pelleted, resuspended in 45 μl of ligation mixture (specified above) and ligated for 6 hours at 15°C to form circular DNA.

Aliquots of 1 μl and 3 μl of the ligation mixture are added to 100 μl of calcium-treated, transformation competent E.coli HB101 cells (prepared according to the method of D.Hanahan [J. Biol. Chem. 166,557(1983)]). The cells are left on ice for 30 min, then incubated for 3 min at 42°C, cooled on ice for 2 min and then incubated for one hour at 37°C in 400 μl of SOC Medium. The cells are concentrated in 100 μl each and plated on LB agar plates containing 50 $\mu\text{g/ml}$ of ampicillin.

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12 transformed, amp^R colonies are grown individually in LB medium containing 100 µg/ml of ampicillin. Plasmid DNA is prepared according to the method of D.S. Holmes et al. [Anal. Biochem. 114, 193 (1981)]. The presence of the synthetic oligonucleotide linker is confirmed by DNA sequencing using a single stranded DNA fragment as primer which hybridizes to the coding strand of hirudin. One clone which contains the linker DNA at the correct position in front of the hirudin gene is referred to as pML310L.

Example 16: Fusion of the PHO5 signal sequence and the desulphatohirudin structural gene

a) Isolation of the 0.2 kb desulphatohirudin fragment (Fig.5)

12 µg of plasmid pML310L are digested to completion with restriction endonucleases BamHI and PvuI. After extraction of the DNA by phenol/chloroform and ethanol precipitation the two restriction fragments are separated on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3. The ethidium-bromide stained 0.84 kb PvuI-BamHI fragment is isolated in a gel slice. The DNA is electroeluted in a dialysis bag filled with 3 ml of 0.2xTBE buffer. The closed bag is submersed in TBE buffer pH 8.3 (90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA). After 30 min at 100 mA the polarity of the current is reversed for 45 sec. to repel the DNA from the dialysis membrane. The buffer surrounding the gel slice in the dialysis bag is recovered, adjusted to 150 mM NaCl and passed through a DE52 (Whatman) ion exchange column. The DNA is eluted with a high salt buffer (1.5 M NaCl, 10 mM Tris•HCl pH 8.0, 1 mM EDTA), precipitated with ethanol and redissolved in H₂O at a concentration of 0.1 mg/ml.

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The 0.84 kb PvuI-BamHI fragment of pML310L is further digested with restriction endonuclease HgaI. This digest generates a 198 bp HgaI-BamHI fragment which contains the complete coding sequence for mature desulphatothrombin. Additional AluI digestion does not touch the 198 bp HgaI-BamHI fragment but eliminates another HgaI fragment of similar size.

The 0.2 kb HgaI-BamHI fragment is separated from other fragments on a 1.5 % agarose gel in TBE buffer and is isolated by electroelution (as described above). The DNA is purified by DE52 ion exchange chromatography and ethanol precipitation. The DNA is resuspended in H₂O at a concentration of 30 µg/ml (0.2 pmoles/ul).

b) Isolation of the PHO5 promoter region with part of the PHO5 signal sequence (Fig. 6)

Plasmid p31/PHO5-TPA18 (cf. European Patent Application No. 143,081) has the PHO5 promoter and the PHO5 signal sequence fused in frame to a foreign structural gene (t-PA). A 584 bp BamHI-BalI fragment contains the PHO5 promoter and all of the PHO5 signal sequence but eight nucleotides at the 3' end.

8 µg of p31/PHO5-TPA18 DNA are digested with restriction endonuclease BalI (16 hours at 37°C). The DNA is purified by phenol/chloroform extraction and ethanol precipitation.

The DNA is resuspended in H₂O at a concentration of 0.7 mg/ml.

The correct junction between the PHO5 signal sequence and the coding region for desulphatohirudin is provided by a synthetic linker of the formula

(1) 5'- CCAATGCA-3'

(2) 3'- GGTTACGTCAACA-5'

Eight nucleotides at the 5' end of the linker (5'-CCAATGCA) represent part of the PHO5 signal sequence from the BalI site to the processing site. The 5' overhanging five nucleotides of oligonucleotide (2) fit into the HgaI cleavage site at the 5' end of the desulphatohirudin coding sequence.

The individual single stranded oligonucleotides (1) and (2) are synthesized by the phosphotriester method (Itakura et al., supra). 1.1 μ g and 1.8 μ g of oligonucleotides (1) and (2), respectively, are individually phosphorylated at their 5' ends, mixed in equimolar amounts and annealed as described in example 15.

1.3 μ g (200 pmoles) of the phosphorylated, double stranded linker DNA is ligated to 7 μ g (1.8 pmoles) of BalI cleaved p31/PHO5-TPA18 in 40 μ l of 60 mM Tris•HCl pH 7.5, 10 mM $MgCl_2$, 3.5 mM ATP, 5 mM DTT and 1400 units of T_4 DNA ligase (Biolabs) at 15°C for 16 hours. The ligase is inactivated for 10 min at 85°C. The excess of linkers is removed by precipitation of the DNA in the presence of 10 mM EDTA, 300 mM sodium acetate pH 6.0 and 0.54 volumes of isopropanol. The DNA is resuspended and further digested with restriction endonuclease BamHI. After extraction of the DNA by phenol/chloroform and

ethanol precipitation the two restriction fragments are separated on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3. The 0.6 kb fragment is isolated from the gel. The DNA is electroeluted and further purified by DE52 ion exchange chromatography and ethanol precipitation. The 0.6 kb BamHI-HgaI DNA fragment is resuspended in H₂O at a concentration of 40 µg/ml.

c) Isolation of a pJDB207 yeast vector fragment (Fig. 6)

9 µg of plasmid pJDB207R/PHO5-TPA (12-2) DNA (cf. European Patent Application No. 143,081) are digested with restriction endonuclease BamHI. Upon complete digestion, the DNA is extracted by phenol/chloroform and ethanol precipitated. The DNA is resuspended in 50 mM Tris•HCl pH 8.0 at a concentration of 0.1 mg/ml and digested with 7 units of calf intestine alkaline phosphatase for one hour at 37°C. The phosphatase is inactivated for 1.5 hours at 65°C and the DNA is purified by DE52 ion exchange chromatography (see example 15) and ethanol precipitation. The 6.8 kb large BamHI fragment is separated on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3. The DNA is electroeluted and purified by DE52 ion exchange chromatography and ethanol precipitation. The DNA is dissolved in H₂O at a concentration of 0.4 mg/ml (0.1 pmoles/µl).

d) Ligation of the PHO5 promoter fragment and the desulphatohirudin structural gene to the pJDB207 yeast vector fragment (Fig. 6)

The pJDB207 yeast vector, the PHO5 promoter fragment with the PHO5 signal sequence and the desulphatohirudin structural gene are all isolated as DNA fragments (see example 16 a-c) which are ligated to form an expression plasmid.

0.3 pmoles of the 0.6 kb BamHI-HgaI fragment of p31/PHO5-TPA18 and 0.3 pmoles of the 0.2 kb HgaI-BamHI fragment of pML310L are ligated to 0.1 pmoles of a 6.8 kb BamHI fragment of pJDB207R/PHO5-TPA(12-2) in 10 μ l of 60 mM Tris•HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP and 400 units of T₄ DNA ligase for 20 hours at 15°C.

A one μ l aliquot of the ligation mixture is added to 100 μ l of transformation competent E.coli HB101 cells prepared according to Hanahan (supra). The transformation protocol is also adopted from this publication. The cells are plated on LB agar plates containing 50 μ g/ml of ampicillin.

24 amp^R colonies are grown individually in LB medium with 100 μ g/ml of ampicillin. Plasmid DNA is analysed for size and orientation of the insert by cleavage with restriction endonuclease PstI. A single clone with the correct orientation of the insert is referred to as pJDB207/PHO5-HIR.

Example 17: Transformation of Saccharomyces cerevisiae strain GRF18

Plasmid pJDB207/PHO5-HIR is introduced into Saccharomyces cerevisiae strain GRF18 (u, his3-11, his3-15, leu2-3, leu2-112, can^R) using the transformation protocol described by Hinnen et al. (supra). 5 μ g of plasmid DNA are added to 100 μ l of a spheroplast suspension and the mixture is treated with polyethylene glycol. The spheroplasts are mixed with 10 ml of regeneration agar and plated onto yeast minimal medium plates without leucine.

A single transformed yeast colony is picked and grown in yeast minimal medium without leucine. The colony is referred to as

Saccharomyces cerevisiae GRF18/pJDB207/PHO5-HIR.

Example 18: Cultivation of *S. cerevisiae* GRF18/pJDB207/
PHO5-HIR:

Cells of *S. cerevisiae* GRF18/pJDB207/PHO5-HIR are agitated in 20 ml of yeast minimal medium (Difco Yeast Nitrogen base without amino acids with the addition of 2 % glucose and 20 mg/l L-histidine) at 30°C and cultivated to a cell density of 3×10^7 cells/ml. The cells are washed in 0.9 % NaCl and then used for inoculating 50 ml cultures in low P_i -minimal medium. Low P_i -minimal medium is prepared corresponding to the composition of Difco Yeast Nitrogen Base medium (without amino acids), but contains 0.03 g/l KH_2PO_4 , 1 g/l KCl, and 10 g/l L-asparagine instead of $(NH_4)_2SO_4$, 2 % glucose and 1 g/l L-histidine. The cultures are inoculated up to a cell density of 4×10^6 cells/ml and agitated at 30°C for up to 42 hours at 200 revs/min.

Example 19: Isolation of the desulphatohirudin compounds
from *S. cerevisiae* GRF18/pJDB207/PHO5-HIR

a) Isolation of desulphatohirudin compounds from the culture medium

A. Enrichment of hirudin compounds on a RP-C18 column

After a fermentation time of 18 h, 26 h and 42 h, respectively, ten samples of 10 ml each are taken from the culture medium and are enriched for proteins by desalting and concentration on a Bond Elut C-18 column (1 ml, Analytichem International). When the samples have been applied to the column and the liquid has been removed in vacuo the column is washed twice with 1.5 ml water-acetonitril (9:1)-0.1% trifluoroacetic acid (9:1). Hirudin compounds are eluted

from the column with water-acetonitril-0.1% trifluoroacetic acid (6:4). 2 ml eluate are concentrated at a Speed Vac concentrator (Savant) to a final volume of 400 μ l. The hirudin compounds are identified by HPLC analysis, by comparison with authentic desulphatohirudin and by means of the thrombin inhibition assay. About 0.2 to 2 μ g/ml hirudin compounds are contained in the samples.

B. Ion exchange chromatography on a DEAE cellulose column

1 l of culture medium is harvested after a fermentation time of 42 h and is centrifuged. The supernatant is applied to an anion exchange column with DE53 cellulose (Whatman) at pH 7.5 [conditions: column 2.5 x 15.5 cm (76 ml); elution buffer A: 20 mM ammonium acetate, 100 mM NaCl, pH 7.5; elution buffer B: 20 mM ammonium acetate, 4M NaCl, pH 5.0; flow : 66 ml/h]. The column is washed with 228 ml buffer A and is then eluted with a linear salt gradient of 228 ml each of buffer A and buffer B, respectively. Fractions of 22 ml are collected. Hirudin compounds are eluted in fractions 71 to 74 (88 ml) and are identified by means of the thrombin inhibition assay. The active fractions are dialysed overnight at 4°C against 20 mM ammonium acetate. Subsequently, aliquots are lyophilised twice. The lyophilisates contain desulphatohirudin compounds as based on reversed phase HPLC.

C. Gel filtration on Sephadex G-50 and final purification by means of HPLC

The main fraction (80 ml of eluate) being active in the thrombin inhibition test, is dialysed overnight at 4°C against 20 mM ammonium acetate pH 7.5 and then concentrated using a rotary evaporator at 35°C to a final volume of 5 ml.

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The resulting clear aqueous solution is applied to a Sephadex G-50 fine column (Pharmacia) with a bed volume of 160 ml, the column (2.5 x 64 cm, Biorad) being pre-equilibrated with 5 % acetic acid. The main activity contained in 50 ml of eluate (fractions 5-7, flow 50 ml/h, 25 min./fraction) is concentrated using a rotary evaporator and then subjected to a reversed phase HPLC separation. An aliquot (500 µl) of the individual fractions is concentrated 1:10 using a "speed vac" concentrator (Savant) and examined by means of reversed phase HPLC analysis for its content of desulphato-hirudin compounds. The solutions contain desulphato-hirudin compounds. Fraction 6 (18 ml) contains the smallest percentage proportion of secondary components and is further purified by means of semipreparative reverse phase HPLC.

Experimental conditions: Vydac 218 TP 510 RP-HPLC column, 10 x 250 mm; aliquot portions of fraction 6 (200 µl concentrated 1:10) per separation; AUFS 0.5 at 220 nm; flow rate: 2 ml/min. Eluant: A: 0.1 % trifluoroacetic acid, B: acetonitrile/water 8:2 + 0.07 % trifluoroacetic acid, 1 min. 16 % B, then increase in the course of 40 min. to 64 % B. The resulting fractions are diluted 1:1 with water and lyophilised. The residue consists of pure desulphatohirudin compounds.

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- b) Isolation of desulphatohirudin compounds from cell extract of *S. cerevisiae*. GRF18/pJDB207/PHO5-HIR

The cells are disintegrated as usual (cf. European Patent Application No. 100,561). The supernatant which has been treated with 2 % acetic acid is centrifuged (12'000 rpm, 10°C). Ammonia is added to a final pH of 7.5 and the slightly opaque solution (50 ml) is further treated as above (cf. example 19a). Hirudin compounds are identified as above, i.e. with the thrombin inhibition assay and by means of reversed phase HPLC.

Example 20: Analysis of the product mixture from the fermentation of *S. cerevisiae* GRF18/pJDB207/PHO5-HIR

a) A 2ml aliquot of the supernatant prepared as above (cf. Example 19b) is dried on a "Speed Vac" concentrator under high vacuum. The samples are dissolved individually in 100 µl of water and subjected to HPLC analysis (experimental conditions no.1, see below). The thrombin inhibition of the individual fractions is tested. The fractions with a retention time of 16.5 min and 17.7 min show thrombin inhibition. The ratio (expression yield) of these two fractions is approximately 4:1. According to amino acid composition these fractions are desulphatohirudins.

b) The fractions active in the thrombin inhibition test obtained as described in Example 19a are subjected to HPLC analysis under two different sets of conditions.

- Experimental conditions No. 1: Nucleosil (MN) C18, 5 µm RP-HPLC column, 4.6 x 120 mm: 50 µl hirudin compounds per separation, att. 6 at 214 nm; flow rate 1.2 ml/min.; eluant: A: 0.1 % trifluoroacetic acid, B: acetonitrile/water 8:2 with 0.08 % trifluoroacetic

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acid, 2 min. 16 % B, then increase in the course of 50 min. to 64 % B. Counterpressure: 66 bars.

- Experimental conditions No. 2: as above, except:
gradient 2 min. 20 % B, then increase in the course of 40 min. to 80 % B.

Fractions are taken at one minute intervals (a total of 45) and subjected to the thrombin inhibition test. Fractions 17-19 prove active. Furthermore, the amino acid composition, the N-terminus and the N-terminal sequence are determined for the active hirudin compounds of fractions 17 and 18. One fraction (No. 17) is in addition compared with authentic desulphatohirudin, which is obtained by reacting natural hirudin from leeches with the enzyme arylsulphatase. The results are compiled in the following table 1:

Table 1:

Fraction	Retention time (min.) Conditions no.1	Retention time (min.) Conditions no.2	Thrombin inhibition (%)
Fr. 17	16.5	13.7	96
Fr. 18	17.7	14.7	86
Fr. 19	18.7	-	78
authentic desulpha- tohirudin	16.5	13.6	86

The ratio in yield of fractions 17 and 18 is again about 4:1.

c) 200 ml aliquots of the supernatant prepared as above (cf. Example 19a) are subjected to HPLC analysis (conditions Nr. 3 see below) and FPLC separation (conditions Nr. 4). The thrombin inhibition of the individual fractions is tested. The fractions with a retention time of 8.0 min (Peak 1),

11.1 min (Peak 2) and 12.1 (Peak 3) have a strong thrombin inhibition. The ratio (expression yield) of these three compounds is approximately 1:4:1. According to HPLC analysis the compounds corresponding to peaks 2 and 3 are identical with the hirudin compounds described in Example 20b) (fractions 17 and 18). The results are summarized in Table 2:

Table 2:

Fraction	Retention time (min) Conditions No 1	Retention time (min) Conditions No 2	Retention time (min) Conditions No 3	[c] NaCl Conditions No 4
Peak 1	14.0	11.6	8.0	365 mM
Peak 2 (Fr.17)	16.5	13.7	11.1	340 mM
Peak 3 (Fr.18)	17.7	14.7	12.1	360 mM

-Experimental conditions No 3: Vydac 218 TP-B5 RP-HPLC column, 4.0 x 120 mm, 15 µg hirudin compound per separation, att. 7 at 214 nm, flow rate 1.2 ml/min; eluant A: 0.1 % trifluoroacetic acid, B: acetonitrile with 0.08 % trifluoroacetic acid, 2 min 16 % B, then increase in the course of 20 min to 40 % B; back pressure 80 bar.

-Experimental conditions No 4: Pharmacia Mono Q FPLC anion exchange-column, 5.0 x 50 mm, 15 µg hirudin compound per separation, AUFS 0.01 at 280 nm, flow rate 1.0 ml/min; gradient elution buffer A: 20 mM Tris-HCl pH 7.5, buffer B: buffer A and 1 M NaCl pH 7.5, 9 min 15 % B, then increase in the course of 21 min to 70 % B.

Example 21: Characterisation of desulphatohirudin compounds from the fermentation of the strain S. cerevisiae GRF 18/pJDB207/PHO5-HIR

According to HPLC analysis the hirudin compounds of fractions 17 (or peak 1) and 18 (or peak 2) isolated from the medium (cf. example 20, Tables 1 and 2) are identical to the corresponding compounds isolated from the cell extracts (intracellular hirudin compounds). These compounds as well as the compound of peak 3 (cf. Table 2) are characterised as follows:

a. Determination of the amino acid composition

Approximately 2.5 µg of the pure hirudin compound are hydrolysed for 24 h with 6N HCl at 110°C and then analysed as described by Chang et al. [DABS-Cl method; Methods in Enzymology 91, 41 (1983)]. The hydrolysate has the following composition:

Peak 1 (cf. Table 2)

Amino acid	Hydrolysate	Amino acid	Hydrolysate
Asp	9.2 (9)	Ile	2.1 (2)
Thr	4.1 (4)	Leu	3.3 (3)
Ser	4.0 (4)	Tyr	1.9 (2)
Glu	12.4 (12)	Phe	1.2 (1)
Pro	3.1 (3)	His	1.0 (1)
Gly	9.0 (9)	Lys	3.1 (3)
Val	3.4 (4)	Met	0 (0)
Cystin	2.5 (3)	Total	(63)

Peak 2 (fraction 17, cf. Tables 1 and 2)

Amino acid	Hydrolysate		Amino acid	Hydrolysate	
Asp	9.9	(9)	Ile	2.1	(2)
Thr	4.0	(4)	Leu	4.4	(4)
Ser	3.9	(4)	Tyr	1.2	(2)
Glu	12.8	(13)	Phe	1.2	(1)
Pro	3.1	(3)	His	1.2	(1)
Gly	9.1	(9)	Lys	2.9	(3)
Val	3.7	(4)	Met	0	(0)
Cystine	2.8	(3)	Total		(65)

Peak 3 (fraction 18, cf. Tables 1 and 2)

Amino acid	Hydrolysate		Amino acid	Hydrolysate	
Asp	10.0	(9)	Ile	1.9	(2)
Thr	3.9	(4)	Leu	3.3	(4)
Ser	4.1	(4)	Tyr	1.8	(2)
Glu	12.5	(12)	Phe	1.1	(1)
Pro	3.1	(3)	His	1.0	(1)
Gly	8.7	(9)	Lys	2.8	(3)
Val	3.1	(4)	Met	0	(0)
Cystin	2.4	(3)	Total		(64)

b) Partial sequence analysis

18 µg (2.5 nmol) of the pure hirudin compound are subjected to a conventional sequence analysis according to Edman and the N-terminal phenylthiohydantoin (PTH)-amino acids are determined by means of RP-HPLC.

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ResultsPeak 2 (fraction 17, cf. Tables 1 and 2)

Cycle 1 5 10
 Amino acid Val Val Tyr Thr Asp n.d. Thr Glu Ser Gly

Cycle 11 15
 Amino acid Gln Asn Leu n.d. Leu n.d. Glu Gly Ser

Circle 20 25
 Amino acid Asn Val n.d. Gly n.d. Gly Asn Lys n.d.

Cycle 29 35
 Amino acid Ile Leu Gly Ser Asp Gly Glu Lys Asn

Cycle 38 40 45
 Amino acid n.d. n.d. Val Thr Gly Glu Gly Thr Pro Lys

Cycle 48 . 50
 Amino acid Pro n.d. Ser n.d.

(n.d. = not determined)

Peak 1 (cf. Table 2)

Sequence from cycle 1 to cycle 29 as above.

Peak 3 (fraction 18, cf. Tables 1 and 2)

Sequence for cycle 1 to cycle 27 as above.

The N-terminal sequences of the biosynthetic hirudin compounds examined are thus identical to that of authentic hirudin.

C. C-terminal analysis

The hirudin compounds are digested with carboxypeptidase Y and the released amino acids determined in the amino acid analyser (cf. J.Y. Chang, R. Knedel, D.G. Braun, Biochem J. 199, 547).

ResultsPeak 1 (cf. Table 2)

C-terminal amino acids: -Glu-Tyr

Peak 2 (fraction 17, cf. Tables 1 and 2)

C-terminal amino acids: -Glu-Tyr-Leu-Gln

Peak 3 (fraction 18, cf. Tables 1 and 2)

C-terminal amino acids: -Glu-Tyr-Leu

d) Apparent molecular weights

The desulphatohirudin compounds (30 µg) are analysed on a SDS urea gel [SUDS gel; cf. Kyte et al., Anal. Biochem. 133, 515 (1983)]. Prior to Coomassie blue staining fixation is done with 12 % trifluoroacetic acid. A single band corresponding to a apparent molecular weight of between 6000 to 7000 Daltons is observed.

e) Molecular weight determination by FAB-MS

The desulphatohirudin compounds are subjected to fast atom bombardment positive ion mass spectrometry (FAB-MS). Instrument: ZAB-HF mass spectrometer from VG-Analytical Ltd., Manchester; matrix: thioglycerol; Xenon bombardment; ion energy 10 KeV; extern calibration: $\text{Cs}_{26}\text{J}_{25}$ (molecular weight: 6387.9) and $\text{Cs}_{28}\text{J}_{27}$ (7147.76).

ResultsPeak 1 (cf. Table 2)

empirical formula: $C_{276}H_{421}N_{77}O_{107}S_6$
 molecular weight (calculated): 6722.23
 molecular weight (found): 6719.3

Peak 2 (fraction 17, cf. Tables 1 and 2)

empirical formula: $C_{287}H_{440}N_{80}O_{110}S_6$
 molecular weight (calculated) 6963,518
 molecular weight (found) 6963,44

The molecular weight is averaged from two different measurements.

Peak 3 (fraction 18, cf. Tables 1 and 2)

empirical formula: $C_{282}H_{432}N_{78}O_{108}S_6$
 molecular weight (calculated): 6835.39
 molecular weight (found): 6832.9

Based on the presented data the compound of peak 1 is desulphatohirudin (1-63), the compound of peak 2 is identical with authentic desulphatohirudin (1-65) and the compound of peak 3 is novel desulphatohirudin (1-64).

f) Human thrombin-hirudin dissociation constants

The dissociation constants K_D of the human thrombin-desulphatohirudin complexes are determined according to the method of S.R. Stone and J.Hofsteenge, [Biochemistry 25, 4622-4628 (1986)] and are compiled in the following table. The table contains also the specific activities (expressed in ATU/mg) of the three desulphatohirudin species obtained.

	K_D [M]	Specific activity [ATU/mg]
yeast desulphatohirudin (1-65)	2.9×10^{-13}	11000
yeast desulphatohirudin (1-64)	3.9×10^{-13}	9600
yeast desulphatohirudin (1-63)	3.0×10^{-12}	8900

g) Further desulphatohirudin compounds obtained from transformed yeast

The mixture of desulphatohirudin compounds, fermented and purified as described in Example 19 is further separated and in detail characterized.

The semipreparative separation on the Vydac 218 TP 510 RP-HPLC column yields in addition to the desulphatohirudin compounds described above (cf. Example 21e) desulphatohirudin-like compounds eluting from the column with the retention time 8.1 minutes.

This material is characterized by fast atom bombardment mass spectrometry (FAB-MS) and amino acid analysis, partial sequencing, and C-terminal analysis.

FAB-MS reveals that the material is a mixture of two compounds having a molecular weight of 6429.5 and 6556.8, respectively.

The amino acid composition is as follows:

<u>Amino acid</u>	<u>Hydrolysate</u>	<u>Amino acid</u>	<u>Hydrolysate</u>
Asx	8.9 (9)	Ile	2.1 (2)
Thr	3.7 (4)	Leu	3.6 (3)
Ser	4.1 (4)	Tyr	0.9 (1)
Glx	10.7 (11)	Phe	1.1 (1)
Pro	3.2 (3)	His	1.2 (1)
Gly	9.7 (9)	Lys	3.2 (3)
Val	3.3 (4)	Met	0 (0)
Cystin	3.3 (3)	Total	(61)

Partial sequence analysis (N-terminus)

Cycle	1	2	3
Amino acid	Val	Val	Tyr

C-terminal analysis (cf. Example 21c)

	59	60	61	62
C-terminal amino acids	-Ile-Pro-Glu-(Glu)			

Based on these data this mixture consists of desulphato-hirudin (1-61) and of desulphatohirudin (1-62).

Example 22: Expression of desulphatohirudin compounds in yeast harboring a desulphatohirudin gene without signal sequence. (Fig.7)

a) Isolation of the pJDB207 vector fragment;

6 ug of the plasmid pJDB207R/IF(α -3) (EP 100,561) are completely digested with the restriction endonuclease BamHI. The resulting DNA fragments (6.85 kb

and 1.15 kb) are precipitated with ethanol and taken up in 400 μ l of 50 mM tris.HCl pH 8.0. 4.5 units of calf intestinal phosphatase (Boehringer, Mannheim) are added. The mixture is incubated for 1 hour at 37°C, then the phosphatase is inactivated at 65°C for 1.5 hours. The solution is adjusted to 150 mM NaCl and then applied to a DE52 (Whatman) anion exchange column (100 μ l volume) which has been equilibrated beforehand with 10 mM tris.HCl pH 7.5, 150 mM NaCl, 1mM EDTA. After a washing operation with the same buffer, the DNA is eluted with 400 μ l of 1.5M NaCl, 10 mM tris.HCl pH 7.5, 1 mM EDTA and precipitated with ethanol. The large 6.85 kb BamHI fragment is separated from the small fragment on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3.

b) Isolation of the 534 bp PH05 promoter fragment

6 μ g of the plasmid p31/R (EP 100,561) are digested with the restriction endonucleases EcoRI and BamHI. The resulting 3 DNA fragments are separated on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3. The 534 bp BamHI-EcoRI fragment is isolated. It contains the PH05 promoter including the transcription start sites.

c) Isolation of a 206 bp DNA fragment with the sequence coding for desulphatohirudin

9 μ g of the plasmid pML310 (cf. Example 13c) are completely digested with the restriction enzymes BamHI and EcoRI. The resulting two DNA fragments are separated on a 1.2 % agarose gel in tris-borate-EDTA buffer pH 8.3. The 206 bp EcoRI-BamHI fragment is isolated.

d) Ligation of the DNA fragments

The three DNA fragments mentioned in section a-c (see above) are obtained from agarose gel blocks by

electroelution, purified over DE52 (Whatman) anion exchangers, precipitated with ethanol and resuspended in H₂O.

0.1 pmol (0.45 µg) of the 6.85 kb BamHI vector fragment, 0.3 pmol (0.1 µg) of the 534 bp BamHI-EcoRI PH05 promoter fragment and 0.25 pmol (34 ng) of the 206 bp EcoRI-BamHI fragment of pML310 are ligated in 15 µl of 60 mM tris.HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP with 600 units of T₄ DNA ligase (Biolabs) at 15°C for 16 hours.

24 transformed amp^R colonies are separately cultivated in LB medium with 100 µg/ml ampicillin. Plasmid DNA is isolated in accordance with the method of Holmes et al. (supra) and analysed by a HindIII-EcoRI double digestion. The appearance of an approximately 600 bp large EcoRI-HindIII fragment indicates that in the clone concerned the PH05 promoter-hirudin DNA fragment is in the correct orientation to the transcription stop signals on the vector. As expected, approximately 50 % of the clones have the correct orientation of the insert. One of these clones is designated pJDB207R/PH05-HIR.

e) Transformation of *Saccharomyces cerevisiae* GRF18:

Saccharomyces cerevisiae strain GRF18 (α, his3-11, his3-15, leu2-3, leu2-112, can^R) is transformed with the plasmid pJDB207R/PH05-HIR in accordance with the protocol of Hinnen et al. (supra). Selection for transformed yeast cells is carried out on agar plates with yeast minimal medium without leucine. A transformed yeast colony is isolated and designated *Saccharomyces cerevisiae* GRF18/pJDB207R/PH05-HIR.

f) Cultivation of *S. cerevisiae* GRF18/pJDB207R/PH05-HIR:

Cells of *S. cerevisiae* GRF18/pJDB207R/PH05-HIR

are agitated in 20 ml of yeast minimal medium (Difco Yeast Nitrogen base without amino acids with the addition of 2 % glucose and 20 mg/l L-histidine) at 30°C and cultivated to a cell density of 3×10^7 cells/ml. The cells are washed in 0.9 % NaCl and then used for inoculating 50 ml cultures in low P_i -minimal medium. Low P_i -minimal medium is prepared corresponding to the composition of Difco Yeast Nitrogen Base medium (without amino acids), but contains 0.03 g/l KH_2PO_4 , 1 g/l KCl, and 10 g/l L-asparagine instead of $(NH_4)_2SO_4$, 2 % glucose and 1 g/l L-histidine. The cultures are inoculated up to a cell density of 4×10^6 cells/ml and agitated at 30°C for 42 hours at 200 revs/min.

g) Analysis of the product mixture from the fermentation of *S. cerevisiae* GRF18/pJDB207R/PH05-HIR

The cells (cf. Example 22f) are disintegrated as usual (cf., for example, European Patent Application No. 100,561). The supernatant treated with 1.5 % acetic acid is centrifuged and each 2 ml of the clear solution is dried on a "Speed Vac" concentrator and in high vacuum. The samples are dissolved individually in 100 μ l of water and subjected to HPLC analysis (conditions as in Example 20). The thrombin inhibition of the individual fractions is tested. The fraction with a retention time of 16.5 min has thrombin inhibition activity. According to amino acid composition this fraction is desulphatohirudin. Based on HPLC analysis the titer is about 10 μ g/l culture broth. In the medium no hirudin activity is detectable.

The yields in intracellular and extracellular hirudin activity obtained from yeast strains harboring the desulphatohirudin gene without or with an appended signal sequence are summarised in Table 3. The hirudin activity is measured by the thrombin inhibition assay.

Table 3

<u>S. cerevisiae</u> GRF18/plasmid	<u>PHO5</u> signal sequence	hirudin activity ($\mu\text{g/l}$)		total
		culture medium	cell extract	
pJDB207/ <u>PHO5-HIR</u>	+	6×10^3	$<1 \times 10^3$	$7 \cdot 10^3$
pJDB207R/ <u>PHO5-HIR</u>	-	not detectable	10	10

Example 23: Construction of PHO5 promoter deletions

a) Bal31 digestion

Recombinant phage M13mp9/PHO5 Bam-Sal containing the BamHI-SalI fragment of PHO5 depicted in Figure 8 is used as a source for the PHO5 promoter (see European Patent Application No. 143,081).

20 μg of the phage DNA (RF: replicative form) are digested with restriction endonuclease SalI, resulting in a linear DNA of approximately 9 kb. After extraction with phenol/chloroform, the DNA is precipitated with ethanol. The DNA is resuspended in 10mM Tris pH 8.0 at a concentration of 0.5 $\mu\text{g/ml}$. 16 μg of SalI cleaved DNA are digested with 2 U of exonuclease Bal31 (BRL) in 100 μl of 20 mM Tris pH 8.0, 199 mM NaCl, 12 mM MgCl_2 , 12 mM CaCl_2 and 1 mM EDTA. Aliquots of 2 μg DNA each are withdrawn after 1, 2, 3, 4, 5 and 6 min. of incubation at 30°C and are immediately mixed with 50 μl phenol and 60 μl TNE. After extraction with phenol/chloroform and ethanol precipitation, the DNA is resuspended in 10 mM Tris pH 8.0 at a concentration of 100 $\mu\text{g/ml}$. To analyse the extent of exonucleolytic cleavage by Bal31 0.5 μg of DNA from each time point are digested with endonuclease BamHI and analysed on a 1.5 % agarose gel in Tris-borate buffer pH 8.3 (90 mM Tris•

HCl pH 8.3, 90 mM boric acid, 2.5 mM EDTA). On the average 100 bp are removed from each end of the fragment per 1 min of Bal31 digestion.

b) Addition of EcoRI linkers to the Bal31 treated DNA

Two A₂₆₀ units of EcoRI linkers (5'-GGAATTCC-3', BRL) are re-suspended in 250 µl of 10 mM Tris pH 8, 1mM EDTA. Two µg of EcoRI linkers are kinased in 75 µl of 60 mM Tris pH 7.5, 10 mM MgCl₂, 15 mM DDT, 10 µM ATP and 33 U of T4 polynucleotide kinase (Boehringer). After 1 h at 37°C the mixture is allowed to cool to room temperature and is then stored at -20°C.

The annealed, double stranded EcoRI linkers are ligated with their blunt ends to the Bal31 treated DNA fragments. Half a microgram of Bal31 treated DNA (see Example 23a) is incubated for 16 hours at room temperature with a 50fold excess of kinased EcoRI linkers in 20 µl of 60 mM Tris pH 7.5, 10mM MgCl₂, 10 mM DTT, 4 mM ATP and 600 U of T4 DNA ligase (Bio-labs). After inactivation of the T4 DNA ligase (10 min at 65°C) the excess of EcoRI linkers is cleaved by 50 U of EcoRI (Boehringer) in a volume of 50 µl. The DNA is extracted with phenol/chloroform, precipitated by ethanol and resuspended in 10 mM Tris, 1 mM EDTA (=TE). Then, the DNA is cleaved with 5 units of BamHI (Biolabs) and the mixture is applied to a 1.5 % low melting agarose gel (Sigma) in Tris-borate buffer (see above). The bands are stained with ethidium bromide and visualized under long wave UV light at 366 nm. The broad diffuse banding patterns between about 100 bp to 600 bp is cut out of the gel and the DNA is extracted as follows: The piece of agarose is liquified at 65°C, adjusted to 500 mM NaCl and incubated at 65°C for 20 min. One volume of phenol (equilibrated with 10 mM Tris•HCl pH 7.5, 1 mM EDTA, 500 mM NaCl) is added. The aqueous phase is reextracted twice with phenol and once with chloroform. The DNA is preci-

pitated with 2.5 volumes of cold absolute ethanol and collected by centrifugation. The DNA pellet is washed with cold 80 % ethanol and then dried in vacuum. The DNA is resuspended in 10 μ l TE.

c) Ligation into M13mp9

3 μ g of RF of M13mp9 is digested with 15 units EcoRI (Biolabs) and 15 units BamHI (Boehringer) in a volume of 50 μ l. After phenol extraction and ethanol precipitation the DNA is resuspended in 50 μ l TE. Five μ l of cut vector DNA (about 200 ng) are mixed with 10 μ l of the above samples (DNA fragments resulting from various Bal31 digests as described in Example 23b) and ligated in a total volume of 20 μ l in the presence of 60 mM Tris/HCl pH 7.5, 6 mM MgCl₂, 10 mM DTT, 1 mM ATP and 200 U of T4 DNA ligase for 15 hours. Transduction of competent cells of the strain E.coli JM101 is done according to the manual "M13 cloning and sequencing system" published by New England Biolabs. Phages from a number of white plaques are grown and analyzed for the size of their DNA inserts by cleavage with restriction enzymes EcoRI and BamHI.

d) Determination of Bal31 deletion end points by Sanger sequencing (deletions from the SalI site)

Sequencing is done using the dideoxy DNA sequencing system of Sanger et al. [Proc. Natl. Acad. Sci. USA 74, 5463(1977)] as described in the above mentioned manual. The deletion end points are given below:

clone position of last nucleotide of
the PH05 sequence (see fig. 8)

A	-502
B	-471
C	-422
D	-400
E	-392
F	-369
G	-350
H	-328
I	-300
K	-283
L	-255
M	-226
N	-211
O	-187
P	-111
Q	- 88
R	- 57
S	- 33

e) Determination of Bal31 deletion end points by Sanger sequencing (deletions from the BamHI site)

A similar set of Bal31 deletions is done as described under a-c, except that M13mp9 PH05 Bam-Sal is cut by BamHI. The Bal31 digested molecules are cut by EcoRI and SalI, and the generated fragments are cloned into M13mp9 digested with EcoRI and SalI. The deletion end points are given below:

- 90 -

clone position of last nucleotide of
the PHO5 sequence (see fig. 8)

A'	-24
B'	-35
C'	-41
D'	-48
E'	-74
F'	-89
G'	-93
H'	-97
I'	-124
K'	-162
L'	-174
M'	-262
N'	-277
O'	-306
P'	-332
Q'	-346
R'	-361
S'	-382
T'	-393

f) Construction of internal PHO5 promoter deletions

The Bal31 deletion set described under d) produces a "left arm" PHO5 promoter fragment ending with an EcoRI site and the Bal31 deletion set described under e) produces a "right arm" PHO5 promoter fragment ending with an EcoRI site. By combining "left arms" and "right arms" of various positions internal deletions are created which contain an EcoRI linker segment at the site of the deleted DNA. The individual internal deletions are constructed by cutting the "left arms" and "right arms" from the M13mp9 derivatives by restriction endonucleases EcoRI and BamHI (left arms) or EcoRI and SalI (right arms) and isolating the corresponding fragments via soft agarose gel electrophoresis as described under b). Equimolar amounts

of "left arms", "right arms" and 200 ng BamHI and SalI digested M13mp9 vector DNA are ligated as described under c). After transduction into E.coli JM101 white plaques are picked, RF is produced and analyzed by restriction analysis (BamHI, SalI, EcoRI). The following arms are combined to create specific internal deletions (for numbering of the nucleotides cf. Figure 8):

"left arm"	"right arm"	deletion	from - to	number of nucleotides deleted
A	T'	Δ 7	-501 to -394	108
B	T'	Δ 8	-470 to -394	77
C	T'	Δ 9	-421 to -394	28
D	S'	Δ10	-399 to -383	17
E	R'	Δ11	-391 to -362	30
F	Q'	Δ12	-368 to -347	22
G	P'	Δ13	-349 to -333	17
H	O'	Δ14	-327 to -307	21
I	N'	Δ15	-299 to -278	22
K	M'	Δ16	-282 to -263	20
L	L'	Δ17	-254 to -175	80
M	L'	Δ18	-225 to -175	51
N	L'	Δ19	-210 to -175	36
O	L'	Δ20	-186 to -175	12
O	K'	Δ21	-186 to -163	24
O	I'	Δ22	-186 to -125	62
O	H'	Δ23	-186 to - 98	89
P	H'	Δ24	-110 to - 98	13
P	G'	Δ25	-110 to - 94	17
P	F'	Δ26	-110 to - 90	21
Q	E'	Δ27	- 87 to - 75	13
R	D'	Δ28	- 56 to - 49	8
R	C'	Δ29	- 56 to - 42	15
R	B'	Δ30	- 56 to - 36	21
S	A'	Δ31	- 32 to - 25	8

g) In vivo analysis of the internal deletions of the PHO5 promoter

The various deletions described in Example 23f) are cloned into plasmid pJDB207/PHO5 [R.Haguenauer-Tsapis and A. Hinnen, Molecular and Cellular Biology, 4, 2668-2675 (1984)] by replacing the wild type PHO5 Bam-Sal fragment with the deleted version. After transformation of yeast strain S.cerevisiae AH216 (cf. European Patent Application No. 143,081) the acid phosphatase activity is determined as described by Toh-e et al. [J. Bacteriol. 113, 727 (1973)]. Deletions $\Delta 11$ and $\Delta 12$ show about a 10-fold reduction of PHO5 activity and define an upstream region ("upstream activation site", UAS) which is essential for PHO5 expression. A similar down effect is observed for deletion $\Delta 17$ (about 5-fold reduction) and for TATA box deletions $\Delta 23$ - $\Delta 26$ (about 30-fold reduction). All other deletions show activities of approximately wild type level. These results suggest that three areas of essential information for PHO5 expression are located at the following positions:

1. between positions -349 and -383 (UAS1)
2. between positions -225 and -263 (UAS2)
3. between positions - 87 and -125 (TATA box)

DNA fragments containing the UAS1 or UAS2 or UAS1 and UAS2 of PHO5 can be produced from recombinant phage M13mp9/PHO5 Bam-Sal (see above) by cleavage with appropriate restriction endonucleases. UAS1 is contained in a 268 bp BamHI-ClaI fragment having the formula

```
GATCCGAAAGTTGTATTCAACAAGAATGCGCAAATATGTCAACGTATTTGGAAGTCATCTT
ATGTGCGCTGCTTTAATGTTTTCTCATGTAAGCGGACGTCGTCTATAAACTTCAAACGAAG
GTAAAAGGTTTCATAGCGCTTTTCTTTGTCTGCACAAAGAAATATATATTAAATTAGCACGT
TTTCGCATAGAACGCAACTGCACAATGCCAAAAAAGTAAAAGTGATTAAAAGAGTTAA
TTGAATAGGCAATCTCTAAATGAAT,
```

UAS2 is contained in a 100 bp ClaI-BstEII fragment having the formula

CGATACAACCTTGGCACTCACACGTGGGACTAGCACAGACTAAATTTATGATTCTGGTCCC
TGTTTTCGAAGAGATCGCACATGCCAAATTATCAAATTG

and both UAS1 and UAS2 are present in the 368 bp BamHI-BstEII fragment having the formula

GATCCGAAAGTTGTATTCAACAAGAATGCGCAAATATGTCAACGTATTTGGAAGTCATCTT
ATGTGCGCTGCTTTAATGTTTTCTCATGTAAGCGGACGTCGTCTATAAACTTCAAACGAAGG
TAAAAGGTTCATAGCGCTTTTTCTTTGTCTGCACAAAGAAATATATATTAAATTAGCACGT
TTTCGCATAGAACGCAACTGCACAATGCCAAAAAAGTAAAAGTGATTAAAAGAGTTAATT
GAATAGGCAATCTCTAAATGAATCGATACAACCTTGGCACTCACACGTGGGACTAGCACAG
ACTAAATTTATGATTCTGGTCCCTGTTTTCGAAGAGATCGCACATGCCAAATTATCAAATTG.

Example 24: Expression of desulphatohirudin under the control of a fused PHO5 - GAPDH hybrid promoter

Example 23 makes a region around position -365 [UAS1(PHO5)] and another region around position -180 [UAS2(PHO5)] of the PHO5 gene possible candidates for UAS with regulating functions. UAS1(PHO5) is contained in a 268 bp BamHI-ClaI fragment whereas both UAS1(PHO5) and UAS2(PHO5) are contained in a 368 bp BamHI-BstEII fragment. These two fragments are each fused to two different GAPDH downstream promoter elements which include the TATA box and transcription initiation sites of GAPDH.

a) Construction of a yeast gene library

Thirty µg of total high molecular weight yeast DNA [M.V.Olsen et al., J.Mol.Biol. 132, 387(1979)] from wild type Saccharomyces cerevisiae strain S288C is incubated for 30 min at 37°C with 2 units of EcoRI methylase (New England Biolabs) in 250 µl of EcoRI methylation buffer as recommended by the supplier. DNA is precipitated by ethanol, resuspended in 500 µl of 25 mM Tris•HCl pH 8.5, 2 mM MgCl₂ (EcoRI* buffer) [H.Meyer, FEBS Lett. 90, 341 (1979)] and digested with EcoRI

(Boehringer) until the size distribution of the DNA fragments has a maximum in the 30-50 kb range (a XhoI digest of λ DNA provides appropriate 33 kb and 17 kb markers). The yeast DNA digested under EcoRI* conditions is size-fractionated on a sucrose gradient (5-20% sucrose in 10 mM Tris•HCl pH 7.5, 1 mM EDTA) for 6 hrs at 38'000 rpm in a SW 40 rotor. Thirty fractions of 0.4 ml each are collected from the top of the gradient. Fraction 16 contains DNA fragments of 30-40 kb in size. The DNA of this fraction (3 μ g) is precipitated with ethanol and ligated for 16 hours at 15°C in a total volume of 15 μ l to 1 μ g of cosmid vector pYcl [B.Hohn et al. in "Genetic Engineering", Vol. 2, p. 169, New York 1980] linearized by EcoRI. Ligation is carried out with 300 U T4 DNA ligase (New England Biolabs) using the buffer system described by the supplier. The DNA is packaged in vitro into bacteriophage λ [B.Hohn in "Methods in Enzymology", Vol. 68, p. 299, New York 1979] and the assembled phages are used to transduce E.coli strain HB101 (r_k^\ominus , m_k^\ominus , leu $^\ominus$, pro $^\ominus$, recA). The efficiency of transduction is about 5000 ampicillin-resistant colonies per μ g of pYcl vector. 3000 amp^R colonies are picked and grown individually in the wells of microtiter dishes in LB medium [10 g Bacto-Tryptone (Difco), 5 g Bacto Yeast Extract (Difco), 10 g NaCl] containing 100 μ g/ml ampicillin.

b) Isolation of the yeast GAPDH gene

The gene library described above is screened with a synthetic oligonucleotide [prepared using the phosphotriester method: K.Itakura et al., J.Am.Chem. Soc. 97, 7327 (1975); J.F.M. de Rooij et al., Recl. Trav. Chim. Pays-Bas 98, 537 (1979)] of the following structure: 5'-GCTCCATCTTCCACCGCCCC-3'. 10 μ g of the oligonucleotide are kinased using 10 μ l of γ -³²P-ATP (3000 Ci/mmol, 10 μ Ci/ μ l Amersham) with T4 polynucleotide kinase (Boehringer) in a total volume of 50 μ l as described by Maniatis et al. ["Molecular Cloning", Cold Spring Harbor Lab., 1982, page 125]. Colony hybridization is performed as

described by the same author (page 312). Positive clones are detected by autoradiography using Kodak X-5 X-ray film. Plasmid DNA isolation produces a hybrid clone which contains a 2100 bp HindIII fragment coding for GAPDH [J.P. Holland et al., J.Biol.Chem. 254, 9839 (1979)]. Final proof for the authenticity of the cloned DNA comes from DNA sequencing experiment using the above mentioned oligonucleotide in combination with the dideoxy sequencing protocol as described by G.F. Hong [Bioscience Reports 1, 243 (1981)] for double stranded DNA. The cloned GAPDH gene (see Fig. 9) has the same sequence as pgap491 published by Holland et al. [J.Biol.Chem. 255, 2596 (1980)].

c) Preparation of GAPDH downstream promoter elements (cf. Fig. 10)

The 649 bp TaqI fragment which includes position -27 to -675 from the ATG of the GAPDH gene (see Figure 9) is isolated by digesting the above mentioned hybrid plasmid with TaqI (New England Biolabs), separating the DNA fragments on a 1.2 % soft agarose gel and extracting the DNA by hot phenol (see example 23). Cloning of the TaqI fragment is done into the ClaI site of pBR322: 1 µg of pBR322 is cleaved with three units of ClaI (New England Biolabs) as described by the supplier. 300 ng of the phenolized and cut vector is ligated to about 300 ng of insert DNA (649 bp Taq fragment) using 200 U of T4 DNA ligase in a total volume of 20 µl (see example 23b). Transformation is done into E.coli HB101 for ampicillin resistance, plasmid DNA is prepared and analyzed by restriction analysis [TaqI, DraI]. The orientation of the TaqI fragment is established using restriction endonuclease DraI in combination with the BamHI site of the plasmids and a plasmid is selected which has the TaqI site of position -675 close to the HindIII site of pBR322. This plasmid designated pBR322/GAPDH is linearized using

BamHI (New England Biolabs), and a digestion with Bal31 is performed as described in Example 23 except that BglII linkers (5'-CAGATCTG-3', New England Biolabs) are used, and the digested plasmid is directly circularized at a concentration of 5 µg/ml in a total volume of 20 µl. The size of the Bal31 shortened TaqI fragment is determined by restriction analysis (using BglII and HindIII). Two clones are selected which contain DNA fragments which extend about 200 bp and 265 bp respectively, from the ATG upstream into the GAPDH promoter. Both fragments contain the presumptive TATA box at about -140 bp. These clones still contain the origin of replication in the pBR322 derived part of the DNA and are named pGAPDH-F and pGAPDH-E, respectively.

d) Combining the downstream GAPDH promoter element with UAS1 (PHO5) of PHO5 and the protein coding region of desulphatohirudin

I) The GAPDH element

In order to extend the GAPDH promoter element from the TaqI site at position -27 to a position immediately adjacent to the ATG of the GAPDH gene two synthetic complementary oligonucleotides of the following structure are synthesized:

```

5' CGAATAAACACACATAAATAAAG      3'
3'  TTATTTGTGTGTATTTATTTCTTAA 5'

```

These oligonucleotides provide the genuine GAPDH promoter sequence from position -26 to position -5 with the generation of a terminal EcoRI site. Two µg of the two Bal31 isolates from example 24c) (plasmids pGAPDH-E and -F) are digested with 6 units of TaqI in 50 µl and the resulting mixture is phenolized, ethanol precipitated and resuspended in 10 µl of water. The synthetic oligonucleotides are annealed by mixing 2 µl of each single strand in 100 µl of a solution containing 10mM Tris•HCl pH 7.5, 10 mM MgCl₂, 50 mM NaCl, heating for

3 min. to 90°C and slowly cooling the solution to room temperature (within about 3 hours). One µg of each of the TaqI digested plasmids is mixed with about a twenty fold molar excess of the annealed oligonucleotides in a volume of 20 µl for about 18 hours using 800 U of T4 DNA ligase. The whole mixture is digested with 3 units of BglII (New England Biolabs), the DNA fragments are separated on a 1.5 % soft agarose gel. The BglII-EcoRI fragments of about 200 bp and 265 bp, respectively, are cut from the gel, extracted and ethanol precipitated.

Plasmid pGAPDH-E is digested with BglII and EcoRI and the large (about 3.5 kb) fragment is isolated. This fragment is used as vector to clone the 265 bp and the 200 bp BglII-EcoRI fragments using ligation, transformation and plasmid isolation conditions as described above. The plasmids produced are designated pGAPDH-EL and pGAPDH-FL. The DNA sequences of the BglII-EcoRI fragments cloned in plasmids pGAPDH-EL and pGAPDH-FL are depicted in Fig. 11. The exact size of the fragments is 266 bp and 201 bp, respectively.

II) The UAS1 (PH05) regulatory element

3 µg of plasmid p31/Y (see European Patent Application No. 100,561) are digested with 6 units of ClaI (New England Biolabs). The 3' recessed ends are filled in using the Klenow fragment of E.coli DNA polymerase I (Bethesda Research Laboratories) according to Maniatis (supra). BglII linkers (5'-CAGATCTG-3') are added as described in example 23. The DNA is digested with SalI and BglII (New England Biolabs) and run on a 1 % soft agarose gel. The 548 bp fragment is cut from the gel, phenolized and ethanol precipitated as described above.

III) The DNA coding for desulphatohirudin

Construction of plasmid pJDB207/PHO5(Eco)-HIR (cf. Fig. 12). For convenient joining of the UAS(PHO5)-GAPDH hybrid promoter elements to the coding region of desulphatohirudin including the PHO5 signal sequence (as in plasmid pJDB207/PHO5-HIR) an EcoRI restriction site is introduced in the 5' nontranslated region between the mRNA start sites and the ATG of the coding region.

15 µg of plasmid pJDB207/PHO5-HIR (see Example 16d) are digested with restriction endonuclease DraI (Boehringer). The resulting 4 fragments are separated on a 0.8 % agarose gel in Tris-borate-EDTA buffer pH 8.3. The 4.2 kb DNA fragment is recovered from the gel, electroeluted and precipitated by ethanol. The DNA is resuspended in H₂O at a concentration of 0.6 mg/ml.

Two synthetic oligonucleotides of the formula

5'- AATTCGATTACCAATGTTT -3'

3'- GCTAATGGTTACAAA -5'

(2.3 µg and 2.9 µg, respectively) are each kinased in 20 µl of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.5 mM ATP and 20 U of T4 polynucleotide kinase (Boehringer). After 45 min at 37°C both reaction mixtures are combined, heated for 10 min at 75°C and allowed to cool to room temperature. The annealed oligonucleotide linker is stored at -20°C.

6.5 µg (2.3 pmoles) of the 4.2 kb DraI DNA fragment are incubated for 16 h at 15°C with a 70 fold excess of the kinased and annealed oligonucleotide linker in 50 µl of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 3.5 mM ATP and 800 U of T4 DNA ligase (Biolabs). After inactivation of the T4 DNA ligase for 10 min at 85°C the excess linkers are removed by precipitation of the DNA in the presence of 10 mM EDTA, 300 mM sodium-acetate pH 6.0 and 0.54 volumes of iso-

propanol. The DNA is digested with EcoRI and HindIII. The resulting fragments are separated on a 1 % agarose gel in Tris-borate-EDTA buffer pH 8.3. The 643 bp fragment is recovered from the gel by electroelution and ethanol precipitation. The DNA is resuspended at a concentration of 0.1 pmoles/ μ l. The EcoRI-HindIII fragment contains the PHO5 signal sequence, the coding sequence of desulphatohirudin and the PHO5 transcription terminator.

The 534 bp PHO5 promoter fragment is isolated from plasmid p31/R (European Patent Application No. 100,561).

Ten μ g of p31/R are digested with restriction endonucleases EcoRI and BamHI. The resulting 3 fragments are separated on a 0.6 % low melting agarose gel in Tris-borate-EDTA buffer pH 8.3. A 534 bp BamHI-EcoRI fragment is isolated which contains the PHO5 promoter including the mRNA start sites.

The vector fragment is isolated from plasmid pJDB207/PHO5-HIR (see Example 16d). 6 μ g of this plasmid are digested with BamHI and Hind III. The large 6.5 kb BamHI-HindIII fragment is separated from the small fragment on a 0.6 % low melting agarose gel in Tris-borate-EDTA buffer pH 8.3.

Three DNA fragments described above with the appropriate sticky ends are ligated in the following reaction: 0.2 pmoles (70 ng) of the 534 bp BamHI-EcoRI PHO5 promoter fragment, 0.2 pmoles (85 ng) of the 643 bp EcoRI-HindIII fragment (hirudin coding sequence) and 0.1 pmole (0.4 μ g) of the 6.5 kb BamHI-HindIII vector fragment are ligated in 10 μ l of 60 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 400 U of T4 DNA ligase for 6 h at 15°C. A one μ l aliquot of the ligation mixture is added to 100 μ l of calcium treated, transformation competent E. coli HB101 cells.

12 transformed, amp^R colonies are grown individually in LB medium containing 100 $\mu\text{g}/\text{ml}$ of ampicillin. Plasmid DNA is prepared according to the method of Holmes et al. [Anal. Biochem. 114 (1981) 193] and is analysed by EcoRI and BamHI restriction digests. One clone with the expected restriction fragments is isolated and referred to as pJDB207/PHO5(Eco)-HIR.

IV) Ligation of the UAS1(PHO5)-GAPDH hybrid promoters to
the protein coding region of desulphatohirudin

15 μg of plasmid pJDB207/PHO5(Eco)-HIR (see Example 24 dIII) are digested with EcoRI and HindIII. The DNA fragments are separated on a 1 % agarose gel in Tris-borate-EDTA buffer pH 8.3. The 643 bp fragment is isolated from the gel, electroeluted and precipitated with ethanol. The DNA is resuspended in H_2O at a concentration of 0.1 pmoles/ μl .

6 μg of plasmid pJDB207/PHO5-HIR are digested to completion with restriction endonucleases HindIII and SalI. The large 6.3 kb fragment (vector part) is isolated by soft agarose gel electrophoresis, phenol extraction and ethanol precipitation. The vector DNA fragment is resuspended in H_2O at a concentration of 0.05 pmoles/ μl .

10 μg of plasmid pGAPDH-EL (see Example 24dI) are digested with BglII and EcoRI. The 266 bp BglII-EcoRI fragment is separated on a 1.2 % agarose gel in Tris-borate-EDTA buffer pH 8.3, electroeluted from the gel and precipitated with ethanol. The DNA is resuspended in H_2O at a concentration of 0.3 pmoles/ μl .

0.3 pmoles of the 548 bp SalI-BglII fragment comprising UAS1(PHO5) (Example 24dII), 0.3 pmoles of the 266 bp BglII-EcoRI fragment of pGAPDH-EL, 0.3 pmoles of the 643 bp EcoRI-

HindIII fragment of pJDB207/PHO5(Eco)-HIR and 0.12 pmoles of the 6.3 kb SalI-HindIII vector fragment are ligated in 20 μ l of 60 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 400 U of T4 DNA ligase (Biolabs) for 6 h at 15°C. Aliquots of 1 μ l and 3 μ l of the ligation mixture are added to 100 μ l of calcium treated E.coli HB101 cells. Plasmid isolation from amp^R colonies and restriction analysis with SalI, BglII, EcoRI and HindIII is performed as described above (see Example 24dIII). One positive clone is selected and referred to as pJDB207/PAPEL-HIR(UAS1).

An analogous construction is done with the 201 bp BglII-EcoRI fragment isolated from pGAPDH-FL. One selected plasmid is called pJDB207/PAPFL-HIR(UAS1).

V) Ligation of the UAS1(PHO5)-UAS2(PHO5)-GAPDH hybrid promoters to the protein coding region of desulphatohirudin (see Fig.13)

3 μ g each of plasmids pJDB207/PAPEL-HIR(UAS1) and pJDB207/PAPFL-HIR(UAS1) are digested with BglII. After phenol extraction and ethanol precipitation the 3' recessed ends of the DNA are filled in a reaction with E.coli DNA polymerase (Klenow fragment; Bethesda Research Laboratories) according to Maniatis et al. (supra). The enzyme is inactivated at 70°C for 10 min. The DNAs are further digested with SalI and the large 7.2 kb fragments are isolated by soft agarose gel electrophoresis, phenol extraction and ethanol precipitation. Each fragment is resuspended in H_2O at a concentration of 0.05 pmoles/ μ l. The fragments contain the hirudin coding region, most of the vector sequences and either of two different GAPDH promoter elements isolated from pGAPDH-EL or pGAPDH-FL.

Plasmid p31/Y (European Patent Application No. 100,561) is digested with BstEII, incubated with E.coli DNA polymerase

(Klenow fragment) as described above and cleaved with SalI. The 649 bp fragment is separated on a soft agarose gel and recovered by phenol extraction and ethanol precipitation.

0.3 pmoles of the 649 bp fragment of p31/Y comprising the UAS1-UAS2(PHO5) promoter element and 0.15 pmoles of either of the 7.2 kb fragments are ligated and transformed into E.coli HB101 as described above. Plasmids are prepared from amp^R colonies and analysed by restriction digests. Single clones are selected and their plasmid DNAs are referred to as pJDB207/PAPFL-HIR(UAS1 + UAS2) and pJDB207/PAPFL-HIR(UAS1 + UAS2).

Example 25: GAPDH promoter elements fused to the protein coding sequence of desulphatohirudin

Two GAPDH promoter elements of different length (as described in Example 24dI) are ligated to the protein coding region of desulphatohirudin including the PHO5 signal sequence.

The two elements (266 bp and 201 bp, respectively) comprise the GAPDH TATA box, the mRNA start sites and an AT rich 5'nontranslated region. The nucleotide sequence of both elements is shown in Figure 10.

The 3'ends of the elements are both at position -5 from the ATG of the GAPDH gene followed by an EcoRI recognition site (see oligonucleotide linker introduced in Example 24dI), the 5'ends are at position -198 and -263, respectively, from the ATG with BglII linkers added at these positions.

The BglII-EcoRI promoter fragments are ligated to an EcoRI-HindIII fragment with sequences coding for the PHO5 signal sequence and desulfatohirudin and to a HindIII-BamHI vector fragment.

6 μ g of plasmid pJDB207/PHO5-HIR are digested to completion with restriction endonucleases HindIII and BamHI. The large 6.5 kb fragment (vector part) is separated on a 0.8 % agarose gel in Tris-borate-EDTA buffer pH 8.3, electroeluted from the gel, phenol extracted and precipitated with ethanol. The vector DNA fragment is resuspended in H₂O at a concentration of 0.05 pmoles/ μ l.

0.3 pmoles of the 266 bp BglII-EcoRI fragment of pGAPDH-EL (Example 24d IV), 0.3 pmoles of the 643 bp EcoRI-HindIII fragment of pJDB207/PHO5(Eco)-HIR (Example 24d III) and 0.1 pmoles of the 6.5 kb HindIII-BamHI vector fragment are ligated in 10 μ l of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5mM DTT, 1 mM ATP and 400 U of T4 DNA ligase (Biolabs) for 6 h at 15°C. 1 μ l of the ligation mixture is added to 100 μ l of calcium treated E.coli HB101 cells. Plasmid isolation from amp^R colonies and restriction analysis with EcoRI is performed as described in Example 24d III. One positive clone is selected. Its plasmid DNA is referred to as pJDB207/GAPEL-HIR.

An analogous construction is done with the 201 bp BglII-EcoRI fragment isolated from pGAPDH-FL. The plasmid DNA of one selected clone is called pJDB207/GAPFL-HIR.

Example 26: Construction of expression plasmids with tandem inserts of the coding sequence of secreted desulphatohirudin (see Fig. 14)

The following constructions contain a DNA fragment duplicated in a tandem head to tail arrangement. The duplicated fragment comprises the GAPDH promoter or a hybrid PHO5-GAPDH promoter (as described in Examples 24 and 25, respectively), the POH5 signal sequence, the coding sequence of desulphatohirudin and the PHO5 transcription terminator.

7 µg of plasmid pJDB207/GAPEL-HIR are digested with restriction endonuclease HindIII. The 3'recessed ends are filled in a reaction with E.coli DNA polymerase I (Klenow fragment; Bethesda Research Laboratory) according to Maniatis (supra). 1,85 µg of SalI linker 5'-GGTCGACC-3'(Biolabs) are kinased in 50 µl of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 0.5 mM ATP and 50 U of T4 polynucleotide kinase (Boehringer). After 45 min at 37°C the mixture is heated for 10 min at 75°C and allowed to cool to room temperature. 7 µg (1.5 pmoles) of linearized plasmid pJDB pJDB207/GAPEL-HIR with the HindIII sites converted to blunt ends (see above) are incubated for 16h at 15°C with an 80 fold excess of kinased and annealed Sal linkers in 30 µl of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 3,5 mM ATP and 800 U of T4 DNA ligase (Biolabs). After inactivation of the T4 DNA ligase at 75°C for 10 min the excess linkers are removed by precipitation of the DNA in the presence of 10 mM EDTA, 300 mM sodium acetate pH 6.0 and 0.54 volumes of isopropanol. The DNA is digested with SalI. The resulting fragments are separated on a 1 % agarose gel in Tris-borate-EDTA buffer pH 8.3. The 1.1 kb SalI fragment is recovered from the gel by electroelution, phenol extraction and ethanol precipitation. The DNA is resuspended at a concentration of 0.1 pmoles/µl.

5 µg of plasmid pJDB207/GAPEL-HIR are digested with SalI to completion. After phenol extraction and ethanol precipitation the DNA is resuspended in 100 µl of 50 mM Tris pH 8.0. 4.6 U of calf intestine alkaline phosphatase (Boehringer) are added. After 1h at 37°C the phosphatase is inactivated in the presence of 100 mM NaCl, 5 mM EDTA and 0.5 % SDS for 15 min at 68°C. The DNA solution is applied to a 100 µl bed of DE 52 (Whatman) anion exchanger equilibrated with 10 mM Tris pH 7.5, 150 mM NaCl and 1 mM EDTA. After washing the column with the same buffer, the DNA is eluted with 400 µl of 1.5M

NaCl, 10 mM Tris pH 7.5, 1 mM EDTA and precipitated with ethanol. Linearized plasmid is separated from uncut DNA on a 0.6 % agarose gel in Tris-acetate buffer. The linear 7.3 kb DNA fragment is recovered from the gel, electroeluted, phenol extracted, ethanol precipitated and resuspended at a concentration of 0.1 pmoles/ μ l.

0.2 pmoles of the 1.1 kb SalI fragment and 0.1 pmoles of the linearized vector are ligated in 10 μ l of 60 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP and 200 U of T4 DNA ligase for 16h at 15°C. A one μ l aliquot of the ligation mixture is added to 100 μ l of calcium treated, transformation competent E.coli HB101 cells.

24 transformed, amp^R colonies are grown individually in LB medium containing 100 μ g/ml of ampicillin. Plasmid DNA is prepared according to the method of Holmes et al [Anal. Biochem. 114 (1981) 193] and is analysed by EcoRI restriction digest. One clone with the expected restriction fragments is isolated and referred to as pJDB207/[GAPEL-HIR]D.

Analogous constructions are done with plasmids pJDB207/GAPFL-HIR and pJDB207/PAPFL-HIR(UAS1 + UAS2). The resulting plasmid DNA of one selected clone each is called pJDB207/[GAPFL-HIR]D and pJDB207/[PAPFL-HIR(UAS1 + UAS2)]D.

Example 27: Construction of an expression plasmid with four inserts of the coding sequence of secreted desulphatohirudin

Four copies of a DNA fragment comprising a GAPDH-promoter, the PH05 signal sequence, the coding sequence of desulphatohirudin and the PH05 transcription terminator are inserted in the yeast expression vector in a tandem head to tail arrangement.

10/ug of plasmid pJDB207/[GAPFL-HIR]D (see Example 26) are digested with SalI. The sticky ends of the resulting linear fragment are filled in a reaction with E.coli DNA polymerase I (Klenow fragment, BRL) according to Maniatis et al. (supra). 0.94/ug of HindIII linker [5'-CAAGCTTG-3', Biolabs] are kinased, self-annealed and ligated to the linearized plasmid pJDB207/[GAPFL-HIR]D with the SalI sites converted to blunt ends (see above). Linker ligation and isopropanol precipitation is as described in Example 26. The DNA is subsequently cleaved with HindIII and the resulting 2.2 kb fragment is isolated by electroelution, phenol extraction and ethanol precipitation. The DNA is resuspended at a concentration of 0.1 pmoles/ul.

5/ug of plasmid pJDB207/[GAPFL-HIR]D are digested with HindIII to completion. The DNA is dephosphorylated with calf intestine alkaline phosphatase (Boehringer), purified by DE52 ion exchange chromatography and isolated from an 0.6 % agarose gel by electroelution as described in Example 26.

0.2 pmoles of the 2.2 kb HindIII fragment and 0.1 pmoles of the linearized vector are ligated (supra). A one/ul aliquot of the ligation mixture is added to 100/ul of calcium treated, transformation competent E.coli HB101 cells.

12 transformed, amp^R colonies are grown individually in LB medium containing 100/ug/ml ampicillin. Plasmid DNA is prepared and analysed by SalI, XbaI; AvaI, XbaI double digests and BamHI digests. A 1.1 kb BamHI-junction fragment indicates that the insert is present in a head to tail arrangement with respect to the two copies already on the plasmid. One clone with this orientation of the insert is referred to as pJDB207/[GAPFL-HIR]T.

Example 28: Construction of a yeast expression vector containing the PHO5 promoter, the invertase signal sequence and the desulphatohirudin coding region

A) Synthesis of oligodeoxyribonucleotides for invertase signal sequence:

Four oligodeoxyribonucleotides: I-1, I-2, I-3, I-4 are synthesized by DNA synthesizer (model 380B Applied Biosystems). After deblocking the synthetic fragments are purified on a 12 % polyacrylamide gel containing 8 M urea. Salt-free pure oligodeoxyribonucleotides are obtained using Sep. Pak (Waters Associates). These fragments constitute a duplex which encodes the invertase signal sequence with the frequently used yeast codons.

HindIII

ECORI MetLeuLeuGlnAlaPheLeuPheLeuLeu

I-1 5'AATTCATGCTTTTGCAAGCTTTCCTTTTCCTTTT 3'

I-2 3'GTACGAAAACGTTGAAAGGAAAAGGAAAACCGAC 5'

AlaGlyPheAlaAlaLysIleSerAla

I-3 5'GGCTGGTTTTGTCAGCCAAAATATCTGCATCTTAGCGTC 3'

I-4 3'CAAACGTCGGTTTTATAGACGTAGAAATCGCAGAGCT 5'

HgaI
XhoI

B) Subcloning of the invertase signal sequence

a) Preparation of vector:

1.5 µg of p31R/SS-TPAΔ2 (European Patent Application No. 143,081) is digested with 10 U of EcoRI (Boehringer) in 50 µl of 10 mM Tris.HCl pH 7.5, 6 mM MgCl₂, 100 mM NaCl, 6 mM mercaptoethanol for one hour at 37°C. After adding 1 µl of 2.5 M NaCl, 10 U of XhoI (Boehringer) are added and incubated at 37°C for one hour. The 4.2 kb vector is isolated on a 0.8 % preparative agarose gel. The gel slice is transferred to a Micro Colloidor tube (Sartorius GmbH), covered with 200 µl of TE and electroeluted (electrophoresed at 90 mA for 50 min). The TE solution is collected

and precipitated in 2.5 volumes of absolute ethanol after the addition of 0.1 volume 10 x TNE. The DNA pellet is washed with cold 80 % ethanol and dried in vacuum. The DNA is resuspended in 6 μ l TE (40 pmoles/ μ l).

b) Annealing oligodeoxyribonucleotides (I-1, I-2, I-3, I-4),
kination and ligation with vector

A solution containing 10 pmoles of each of the four deoxyribonucleotides in 10 μ l of 0.5 M Tris.HCl pH 8 is incubated at 95°C for 5 minutes on a water bath. The water bath is slowly cooled to 30°C over a period of 5 hours. To this annealed mixture is added 2 μ l each of 0.1 M MgCl₂, 0.1 M NaCl, 30 mM DTT, 4 mM ATP and 8 U (1 μ l) of polynucleotide kinase (Boehringer). Kination is carried out at 37°C for one hour. The annealed, kinased oligodeoxyribonucleotides and 60 pmoles of the EcoRI-XhoI cut vector (1.5 μ l) are ligated with 400 U (1 μ l) of T4 DNA ligase (Biolabs) at 14°C for 17 hours. The reaction is stopped by incubation at 65°C for 10 min. 10 μ l of this ligation mixture is used for transformation of E.coli HB101 Ca⁺⁺ cells [M. Dagert and S.D. Ehrlich, Gene 56, 23-28 (1979)]. 20 amp^R colonies are picked. DNA is prepared by the quick isolation procedure [D.S. Holmes and M. Quigley, Anal.Biochem. 114, 193-197 (1981)]. DNA is digested with EcoRI and XhoI, radiolabelled at the EcoRI end and analysed on a 6 % polyacrylamide gel containing 8 M urea using radiolabelled pBR322 HaeIII cut DNA as marker. Correct size bands are observed for DNA obtained from all the 20 clones. One clone is grown in 100 ml LB medium containing 100 μ g/ml of ampicillin. Plasmid DNA is isolated and is referred to as p31RIT-12.

c) Joining of the GAPFL promoter element to the invertase
signal sequence:

5 μ g of plasmid p31RIT-12 are digested to completion with SalI and EcoRI. The large, 3.3 kb SalI-EcoRI fragment is

isolated on a 0.6 % agarose gel in Tris-acetate buffer pH 8.2. The DNA is electroeluted from the gel, phenol extracted and ethanol precipitated. 10/ug of plasmid pJDB207/GAPFL-HIR (see Example 25) are digested with SalI and EcoRI. The 477 bp SalI-EcoRI fragment is isolated on a 0.8 % agarose gel in Tris-borate-EDTA buffer pH 8.3. The DNA is electroeluted, phenol extracted and ethanol precipitated. Both isolated DNA fragments are ligated. An aliquot of the ligation mixture is used to transform competent E.coli HB101 cells. Amp^R colonies are grown and the plasmid DNA is analysed by HindIII, SalI double digestion. A plasmid with the correct insert is called p31/GAPFL-IT.

D) Construction of plasmid pJDB207/GAPFL-I-HIR (see Fig. 15)

25/ug of plasmid p31/GAPFL-IT are digested to completion with PstI and SalI. The resulting 676 bp fragment is isolated on a 10 % preparative agarose gel in Tris-borate-EDTA buffer pH 8.3. The DNA is electroeluted, purified by DE52 ion exchange chromatography, ethanol precipitated and resuspended in buffer recommended for HgaI digests at a concentration of 0.1/ug/ul. The SalI-PstI DNA fragment is partially digested with HgaI in the presence of 0.5 units/ug DNA for 60 min at 37 °C. The reaction is stopped by the addition of EDTA to a final concentration of 10 mM. The 534 bp SalI-HgaI fragment is isolated on a 1 % preparative agarose gel. The DNA is electroeluted from the gel, purified by DE52 chromatography, ethanol precipitated and resuspended in H₂O at a concentration of about 0.1 pmoles/ul.

10/ug of plasmid pJDB207/PHO5-HIR (see Example 16) are digested with BalI and HindIII. The resulting 587 bp BalI-HindIII fragment is isolated on a preparative 1 % agarose gel. The DNA is electroeluted, ethanol precipitated and further digested with HinfI.

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1.3 μ g (160 pmoles) each of the following oligodeoxy-nucleotides

5'-CTGCAGTTGTTTACACCGACTGCACCG-3'

3'-CAACAAATGTGGCTGACGTGGCTTA-5'

are kinased and annealed as described in Example 24dIII.

0.6 μ g (1.6 pmoles) of the *Hinf*I digested *Bal*I-*Hind*III fragment (see above) and 160 pmoles of the kinased and annealed linker are ligated for 16 hours at 15°C in 83 μ l of 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, 3.5 mM ATP and 400 units of T4 DNA ligase. After 10 min at 85°C excess linkers are removed by isopropanol precipitation of the DNA (see Example 24dIII). The 584 bp *Hga*I-*Hind*III fragment is isolated on a 1 % preparative agarose gel. The electroeluted, purified and ethanol precipitated DNA is resuspended in H₂O at a concentration of 0.1 pmoles/ μ l.

5 μ g of pJDB207/PHO5-HIR are digested with *Hind*III and *Sal*I and the large, 6.2 kb fragment is isolated.

0.2 pmoles of the 534 bp *Sal*I-*Hga*I fragment,
0.2 pmoles of the 584 bp *Hga*I-*Hind*III fragment and
0.1 pmoles of the 6.2 kb *Sal*I-*Hind*III vector fragment are ligated for 5 hours at 15°C in 10 μ l of 10 mM Tris.HCl pH 7.5, 10 mM MgCl₂, 5 mM DTT, 1 mM ATP and 400 units of T4 DNA ligase. A one μ l aliquot of the ligation mixture is used to transform competent E.coli HB101 cells.

24 transformed, ampicillin resistant colonies are grown individually in LB medium containing 100 μ g/ml ampicillin. Plasmid DNA is prepared and analysed by *Hind*III, *Sal*I double digestion. A single clone with the correct restriction pattern is isolated and referred to as pJDB207/GAPFL-I-HIR. The identity of the invertase signal sequence and the correct fusion with the hirudin coding sequence is confirmed by nucleotide sequencing.

Example 29:a) Transformation of *Saccharomyces cerevisiae* GRF 18:

Saccharomyces cerevisiae strain GRF18 (α , his3-11, his3-15, leu2-3, leu2-112, can^R) is transformed with the plasmids
 pJDB207/PAPEL-HIR(UAS1),
 pJDB207/PAPFL-HIR(UAS1),
 pJDB207/PAPEL-HIR(UAS1 + UAS2),
 pJDB207/PAPFL-HIR(UAS1 + UAS2),
 pJDB207/GAPEL-HIR,
 pJDB207/GAPFL-HIR,
 pJDB207/[GAPEL-HIR]D,
 pJDB207/[GAPFL-HIR]D,
 pJDB207/[PAPFL-HIR(UAS1 + UAS2)]D,
 pJDB207/[GAPFL-HIR]T,
 pJDB207/GAPFL-I-HIR

using the transformation protocol described by Hinnen et al. [Proc.Natl.Acad.Sci. USA 75, 1929 (1978)]. Transformed yeast cells are selected on yeast minimal media plates deficient in leucine. Single transformed yeast colonies are isolated and referred to as

Saccharomyces cerevisiae GRF18/pJDB207/PAPEL-HIR(UAS1),
Saccharomyces cerevisiae GRF18/pJDB207/PAPFL-HIR(UAS1),
Saccharomyces cerevisiae GRF18/pJDB207/PAPEL-HIR(UAS1+UAS2),
Saccharomyces cerevisiae GRF18/pJDB207/PAPFL-HIR(UAS1+UAS2),
Saccharomyces cerevisiae GRF18/pJDB207/GAPEL-HIR,
Saccharomyces cerevisiae GRF18/pJDB207/GAPFL-HIR,
Saccharomyces cerevisiae GRF18/pJDB207/[GAPEL-HIR]D,
Saccharomyces cerevisiae GRF18/pJDB207/[GAPFL-HIR]D,
Saccharomyces cerevisiae GRF18/pJDB207/[PAPFL-HIR(UAS1+UAS2)]D,
Saccharomyces cerevisiae GRF18/pJDB207/[GAPFL-HIR]T,
Saccharomyces cerevisiae GRF18/pJDB207/GAPFL-I-HIR.

b) Fermentation of the transformants

Cells of the S.cerevisiae GRF18 transformants are each grown in 10 ml of yeast minimal medium (Difco Yeast Nitrogen Base without aminoacids to which 2 % glucose and 20 mg/l L-histidine are added) in a 50 ml Erlenmeyer flask with shaking at 30°C for 24 hours to a density of 3×10^7 cells/ml. Yeast transformants containing plasmids with a hybrid PHO5-GAPDH promoter require derepression of the promoter for the expression of desulphatohirudin. The cells are washed in 0.9 % NaCl and used to inoculate 50 ml of a low P_i minimal medium prepared according to the recipe of the Difco Yeast Nitrogen Base medium (without amino acids), but containing 0.03 g/l KH_2PO_4 , 10 g/l L-asparagine instead of $(\text{NH}_4)_2\text{SO}_4$, 2 % glucose and 1 g/l L-histidine. The cultures are inoculated up to a cell density of 4×10^6 cells/ml and agitated at 30°C for up to 24 hours at 200 revs/min. Final densities of 1×10^8 cells/ml are obtained.

Yeast transformants comprising plasmids with the GAPDH promoter express desulphatohirudin constitutively. Cells are grown in a complex medium consisting of (g/l) peptone (5), yeast extract (10), glucose (20), sucrose (40), ammonium sulphate (3), KH_2PO_4 (2), MgSO_4 (0.5), NaCl (0.1), CaCl_2 (0.1), biotin (10 $\mu\text{g/l}$). Approximately $1 \cdot 10^9$ cells/ml are obtained after 48 hours of incubation at 30°C and 200 revs/min.

The amounts of desulphatohirudin (as determined by the thrombin inhibition assay) secreted by some representative transformed yeast strains are compiled in the following table.

Strain <u>Saccharomyces cerevisiae</u> GRF18/	secreted hirudin (mg/l)	time of harvest (hours)
pJDB207/PAPFL-HIR(UAS1)	7	24
pJDB207/PAPFL-HIR(UAS1 + UAS2)	8	24
pJDB207/GAPFL-HIR	41	48
pJDB207/GAPEL-HIR	35	48
pJDB207/[GAPFL-HIR]D	39	48
pJDB207/[GAPEL-HIR]D	31	48

Furthermore, it is determined that at least 90 % of the desulphatohirudin compounds expressed by the yeast transformants is secreted into the culture medium. In order to determine the distribution of desulphatohirudin in the culture broth and inside the cells as a function of the time of harvest, fermentation is run in a 3 l MBR bioreactor (MBR Bioreactor AG, Wetzikon, Switzerland). Strain

Saccharomyces cerevisiae GRF18/pJDB207/GAPFL-HIR is inoculated into 3 l of complex medium (see above) and incubated at 30°C with an agitation rate of 500 revs/min and an aeration rate of 200 l/h. A final density of $5 \cdot 10^9$ cells/ml is reacted. Samples are taken at 18, 24 and 42 hours, respectively, and the content of desulphatohirudin in the culture broth and inside the cells (after disintegration, see Example 22) is determined by the thrombin inhibition assay and by HPLC.

	time (h)	desulphatohirudin (mg/l)	
		thrombin inhibition	HPLC
culture broth	18	60	78
cell interior	18	7	<1
culture broth	24	70	70
cell interior	24	9	<1
culture broth	42	110	105
cell interior	42	9	<1

Example 30: Cultivation of transformants on a 300 l scalea) The inoculation cycle

A number of agar slopes are inoculated from a deep frozen ampoule containing cells of strain Saccharomyces cerevisiae GRF18/pJDB207/GAPFL-HIR. One or more agar slopes are incubated for 3-5 days at 28-30°C. The contents of a single agar slope is inoculated sterily into single 500 ml Erlenmeyer flasks without baffles and containing 100 ml of preculture medium. The flasks are shaken on a rotary shaker with a 50 mm throw at 250 r.p.m. with an incubation temperature of 28°C. After 48 hours, 2 % (v/v) of the contents of these flasks (1st preculture) is inoculated sterily into 2 l Erlenmeyer flasks containing 600 ml of preculture medium. These flasks (2nd preculture) are shaken for 48 hours at 28°C on a rotary shaker at 120 r.p.m. with a 50 mm throw. The content of one of the 2nd preculture flasks (2 % v/v) is introduced sterily into a 50 l stainless steel fermenter containing 30 l of preculture medium (plant preculture). The fermentation conditions for the plant preculture are as follows: 600 r.p.m. (single six-bladed turbine stirrer, 115 mm diameter), 4 baffles; head pressure: 0.3 bar; aeration 1 litre of air per litre of medium per minute, temperature 28°C, 48 hours duration.

The agar medium and the preculture medium are the same except for the presence or absence of agar. The same preculture medium was used for all preculture stages.

Composition of preculture medium (g/l):

yeast nitrogen base (Difco)	8.4
L-asparagine•H ₂ O	11.6
L-histidin	1.0
glucose (separately sterilized)	2.0

Composition of agar medium:

same as preculture medium plus 20 g agar/l.

b) The production fermentation (300 l scale)

5 % (v/v, 15 l) of the grown plant preculture are introduced sterilely into a 600 l fermenter containing 300 l of sterile fermentation production medium. The fermentation conditions for the 300 l production medium are as follows: 450 r.p.m. (single six-bladed stirrer, diameter 230 mm); 4 baffles; head pressure: 0.3 bar; air-throughput: 0-9 hours; 0.25 litres of air per litre of medium per minute, 9 hours-harvest: 1 l of air per litre of medium per minute; temperature: 28°C, duration 24-48 hours. OD₆₀₀ 15-20. Yield: 15-25 mg/l (test methods: HPLC and thrombin tests).

Composition of the production medium (g/l):

peptone	5
yeast extract	10
sucrose	40
(NH ₄) ₂ SO ₄	6
MgSO ₄ •7H ₂ O	1
NaCl	0.1
KH ₂ PO ₄	2
CaCl ₂ •2H ₂ O	0.013.

pH before sterilization ~6.0

antifoam: UCON LB 625 as required.

Example 31: Isolation of desulphatohirudin from the
300 l culture broth

The culture broth (cf. Example 30) having a pH value of 3.3 is cooled to 17°C. The cells are separated by a Westfalia SA-14 de-sludging device (400-600 l/h). The thick sludge is discarded. The slightly turbid supernatant is filtered through a series of Skan filter cartouches (first: pore width 5 µm; second: pore width 1 µm; third: pore width

0.22 μ m), brought to pH 7.5 with NaOH and applied to a DEAE anion exchange column. The column is washed with sodium acetate (20 mM, pH 4.5) and eluted with sodium acetate (20 mM, pH 3.1). The solution (15 l) is brought to pH 7.5 with NaOH and concentrated to a final volume of 1.8 l in a circulation evaporator. An aliquot of 1 l is applied to a Sephadex G-25 column (bed volume: 8 l; the column being equilibrated with water). Fractions containing desulphatohirudin are identified by HPLC. The desulphatohirudin fraction (2.5 l) is concentrated with a rotavap in high vacuum to a final volume of 200 ml and is lyophilized.

An aliquot (2 g) of the raw material is dissolved in 50 ml of water, brought to pH 7.5 with NaOH and applied to a Q Sepharose fast flow column (bed volume: 200 ml). The column is washed with ammonium formate (100 mM, pH 3.9). 25 ml fractions are taken and tested for desulphatohirudin by HPLC. Fraction containing desulphatohirudin (1-65) are pooled (125 ml) and concentrated with a rotavap in high vacuum to a final volume of 10 ml. The concentrated solution is applied to a Sephadex G-25 column (Amicon, the column being equilibrated with water) for desalting. The obtained clear solution (25 ml) is lyophilized. The resulting solid consists of pure desulphatohirudin.

Example 32: Construction of plasmid pJDB207/PH05-EGL:

The nucleotide sequence coding for eglin C, a polypeptide of 70 aminoacids from leech has been synthesized in vitro, subcloned and expressed in E.coli as described in European Patent Application No. 146,785. The exact in frame fusion of the eglin coding sequence to the sequence coding for the PH05 signal peptide is achieved in a completely analogous way as described for hirudin in Examples 15 and 16. A single clone with the correct orientation of the insert in the vector is referred to as pJDB207/PH05-EGL. Saccharomyces

cerevisiae strain GRF18 is transformed as usual. Transformants are selected and cultured as described in Example 29. No eglin C is detectable in the culture broth.

CLAIMS (for the contracting states BE, DE, FR, GB, IT, NL, SE, CH, LU, LI)

1. Method for the production of proteins with hirudin activity comprising culturing under appropriate nutrient conditions an eukaryotic host organism transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and isolating the proteins with hirudin activity from the culture medium and, if desired, isolating optionally additional protein with hirudin activity which is cell associated from the cell interior, and, if desired, converting a resulting protein with hirudin activity into a different protein with hirudin activity.

2. Method according to claim 1 characterized in that the expression control sequence is derived from the genomic DNA of yeast for use in a yeast host.

3. Method according to claim 1 characterized in that the expression control sequence is a hybrid promoter comprising upstream activation sequences of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene.

4. Method according to claim 1 characterized in that the DNA sequence encoding a signal peptide is selected from the group consisting of the signal sequences of

the yeast invertase, a-factor, α-factor, pheromone peptidase, "killer toxin" and repressible acid phosphatase (PHO5) genes and the glucoamylase signal sequence from Aspergillus awamori.

5. Method according to claim 1 for the production of Des-(Leu⁶⁴,Gln⁶⁵)-desulphatohirudin variant HV1 having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr.

6. Method according to claim 1 for the production of Des-(Gln⁶⁵)-desulphatohirudin variant HV1 having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu.

7. Method according to claim 1 for the production of desulphatohirudin variant HV1 having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu Gln.

8. Method according to claim 1 for the production of desulphatohirudin variant HV2 having the formula

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
Ile Leu Gly Ser Asn Gly Lys Gly Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Asn Pro Glu Ser His Asn Asn Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu Gln

9. Method according to claim 1 for the production of desulphatohirudin variant PA having the formula

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
Ile Leu Gly Ser Gln Gly Lys Asp Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Gln Gly Asp Phe
Glu Pro Ile Pro Glu Asp Ala Tyr Asp Glu

10. Method according to claim 1 for the production of des-(Val)₂-desulphatohirudin.

11. A DNA construct containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals.

12. A construct according to claim 11 characterized in that the expression control sequence is derived from a virus.

13. A construct according to claim 11 characterized in that the expression control sequence is derived from the genomic DNA of yeast.
14. A construct according to claim 11 characterized in that the DNA sequence encoding a signal peptide is selected from the group consisting of the signal sequences of the yeast invertase, α -factor, α -factor, pheromone peptidase, "killer toxin" and repressible acid phosphatase (PHO5) genes and the glucoamylase signal sequence from Aspergillus awamori.
15. A construct according to claim 11 containing the PHO5 promoter, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of the PHO5 promoter, and the 3' flanking sequence of the PHO5 gene.
16. A construct according to claim 11 containing the GAPDH promoter, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin, which DNA segment is under transcriptional control of the GAPDH promoter, and the 3' flanking sequence of the PHO5 gene.
17. A construct according to claim 11 containing a hybrid promoter comprising upstream activation site(s) of the yeast PHO5 gene and a downstream promoter element including the TATA box of the yeast GAPDH gene, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin,

which DNA segment is under transcriptional control of the hybrid promoter, and the 3' flanking sequence of the PHO5 gene.

18. A construct according to claim 11 containing the PHO5 promoter, a DNA segment consisting of the invertase signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of the PHO5 promoter, and the 3' flanking sequence of the PHO5 gene.

19. Method for the preparation of a DNA construct according to claim 11 comprising linking an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for desulphatohirudin, and a DNA sequence containing eukaryotic transcription termination signals in such a way that proper expression of the DNA segment and secretion of the produced desulphatohirudin is effected in an eukaryotic host organism.

20. A hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals.

21. A hybrid vector according to claim 20 containing a DNA construct according to any one of claim 11 to 18.
22. Method for the preparation of a hybrid vector according to claim 20 comprising introducing one or multiple DNA constructs each containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, as such or introducing the components of said DNA constructs successively in the predetermined order into a vector DNA.
23. A eukaryotic host organism transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and mutants thereof.
24. A eukaryotic host organism according to claim 23 transformed with a hybrid vector according to any one of claims 20 and 21.
25. A transformed eukaryotic host organism according to claim 23 selected from a mammalian cell line and yeast.

26. Method for the production of a transformed eukaryotic host organism according to claim 23 comprising transforming eukaryotic host cells with a hybrid vector having one or multiple DNA inserts each comprising a eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and, if desired, mutating the transformed eukaryotic host cells obtained.

27. Des-(Gln⁶⁵)-desulphatohirudin variant HV1.

CLAIMS (for the contracting states AT,ES,GR)

1. Method for the production of proteins with hirudin activity comprising culturing under appropriate nutrient conditions an eukaryotic host organism transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and isolating the proteins with hirudin activity from the culture medium and, if desired, isolating optionally additional protein with hirudin activity which is cell associated from the cell interior, and, if desired, converting a resulting protein with hirudin activity into a different protein with hirudin activity.

2. Method according to claim 1 characterized in that the expression control sequence is derived from the genomic DNA of yeast for use in a yeast host.

3. Method according to claim 2 characterized in that the expression control sequence is a hybrid promoter comprising upstream activation sequences of one yeast gene and downstream promoter elements including a functional TATA box of another yeast gene.

4. Method according to claim 1 characterized in that the DNA sequence encoding a signal peptide is selected from the group consisting of the signal sequences of

the yeast invertase, a-factor, α-factor, pheromone peptidase, "killer toxin" and repressible acid phosphatase (PHO5) genes and the glucoamylase signal sequence from Aspergillus awamori.

5. Method according to claim 1 characterized in that there is used yeast transformed with a hybrid vector having one or multiple DNA inserts each comprising the PHO5 promoter, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin, which DNA segment is under transcriptional control of the PHO5 promoter, and the 3' flanking sequence of the PHO5 gene.

6. Method according to claim 1 characterized in that there is used yeast transformed with a hybrid vector having one or multiple DNA inserts each comprising the GAPDH promoter, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin, which DNA segment is under transcriptional control of the GAPDH promoter, and the 3' flanking sequence of the PHO5 gene.

7. Method according to claim 1 characterized in that there is used yeast transformed with a hybrid vector having one or multiple DNA inserts each comprising a hybrid promoter comprising upstream activation site(s) of the yeast PHO5 gene and a downstream promoter element including the TATA box of the yeast GAPDH gene, a DNA segment consisting of the PHO5 signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin, which DNA segment is under transcriptional control of the hybrid promoter, and the 3' flanking sequence of the PHO5 gene.

8. Method according to claim 1 characterized in that there is used yeast transformed with a hybrid vector having one or multiple DNA inserts each comprising the PHO5 promoter, a DNA segment consisting of the invertase signal sequence upstream of and in reading frame with a DNA sequence coding for mature desulphatohirudin, which DNA segment is under transcriptional control of the PHO5 promoter, and the 3' flanking sequence of the PHO5 gene.

9. Method according to claim 1 for the production of Des-(Leu⁶⁴, Gln⁶⁵)-desulphatohirudin variant HVI having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr.

10. Method according to claim 1 for the production of Des-(Gln⁶⁵)-desulphatohirudin variant HVI having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu.

11. Method according to claim 1 for the production of desulphatohirudin variant HV1 having the formula

Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys Cys
Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu Gln.

12. Method according to claim 1 for the production of desulphatohirudin variant HV2 having the formula

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
Ile Leu Gly Ser Asn Gly Lys Gly Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Asn Pro Glu Ser His Asn Asn Gly Asp Phe
Glu Glu Ile Pro Glu Glu Tyr Leu Gln

13. Method according to claim 1 for the production of desulphatohirudin variant PA having the formula

Ile Thr Tyr Thr Asp Cys Thr Glu Ser Gly Gln Asn Leu Cys
Leu Cys Glu Gly Ser Asn Val Cys Gly Lys Gly Asn Lys Cys
Ile Leu Gly Ser Gln Gly Lys Asp Asn Gln Cys Val Thr Gly
Glu Gly Thr Pro Lys Pro Gln Ser His Asn Gln Gly Asp Phe
Glu Pro Ile Pro Glu Asp Ala Tyr Asp Glu

14. Method according to claim 1 for the production of des-(Val)₂-desulphatohirudin.

15. Method for the preparation of a DNA construct containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide

upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals comprising linking an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for desulphatohirudin, and a DNA sequence containing eukaryotic transcription termination signals in such a way that proper expression of the DNA segment and secretion of the produces desulphatohirudin is effected in an eukaryotic host organism.

16. Method for the preparation of a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals comprising introducing one or multiple DNA constructs each containing an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphatohirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, as such or introducing the components of said DNA constructs successively in the predetermined order into a vector DNA.

17. Method for the production of a eukaryotic host organism transformed with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphato-hirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and mutants thereof comprising transforming eukaryotic host cells with a hybrid vector having one or multiple DNA inserts each comprising an eukaryotic expression control sequence, a DNA segment consisting of a first DNA sequence encoding a signal peptide upstream of and in reading frame with a second DNA sequence coding for mature desulphato-hirudin which DNA segment is under transcriptional control of said expression control sequence, and a DNA sequence containing eukaryotic transcription termination signals, and, if desired, mutating the transformed eukaryotic host cells obtained.

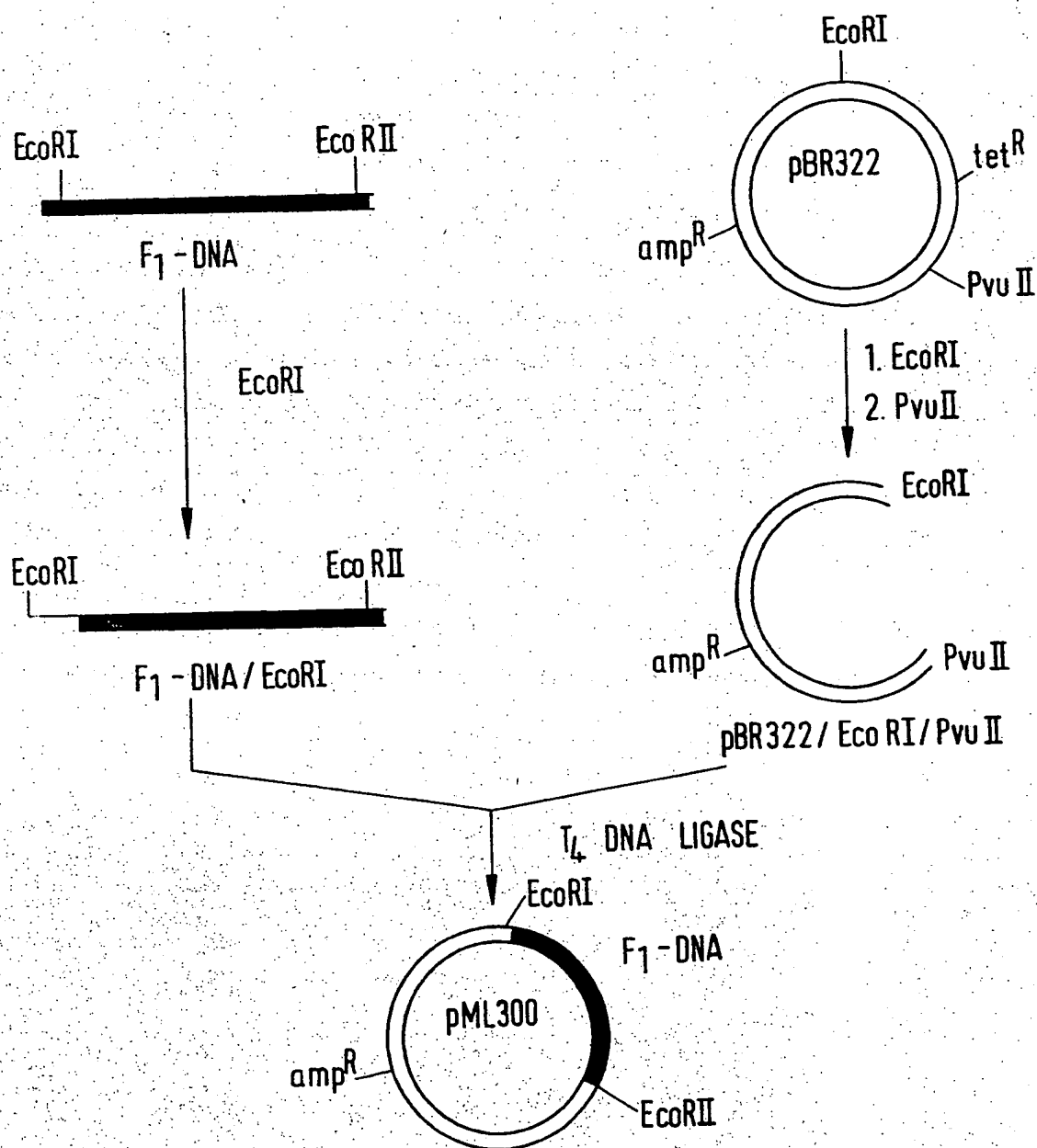
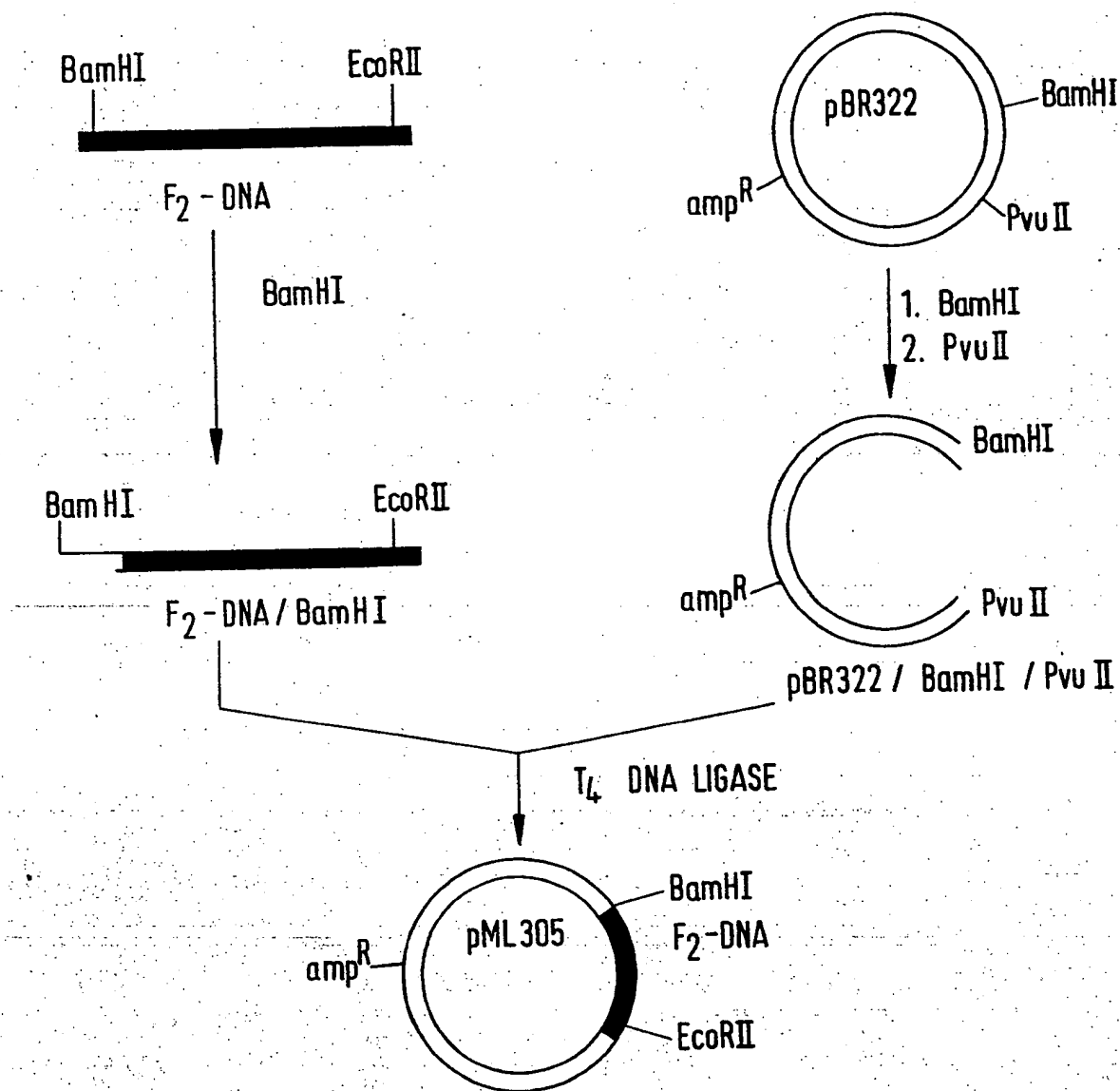
Fig. 1CONSTRUCTION OF PLASMID pML300

Fig. 2CONSTRUCTION OF PLASMID pML305

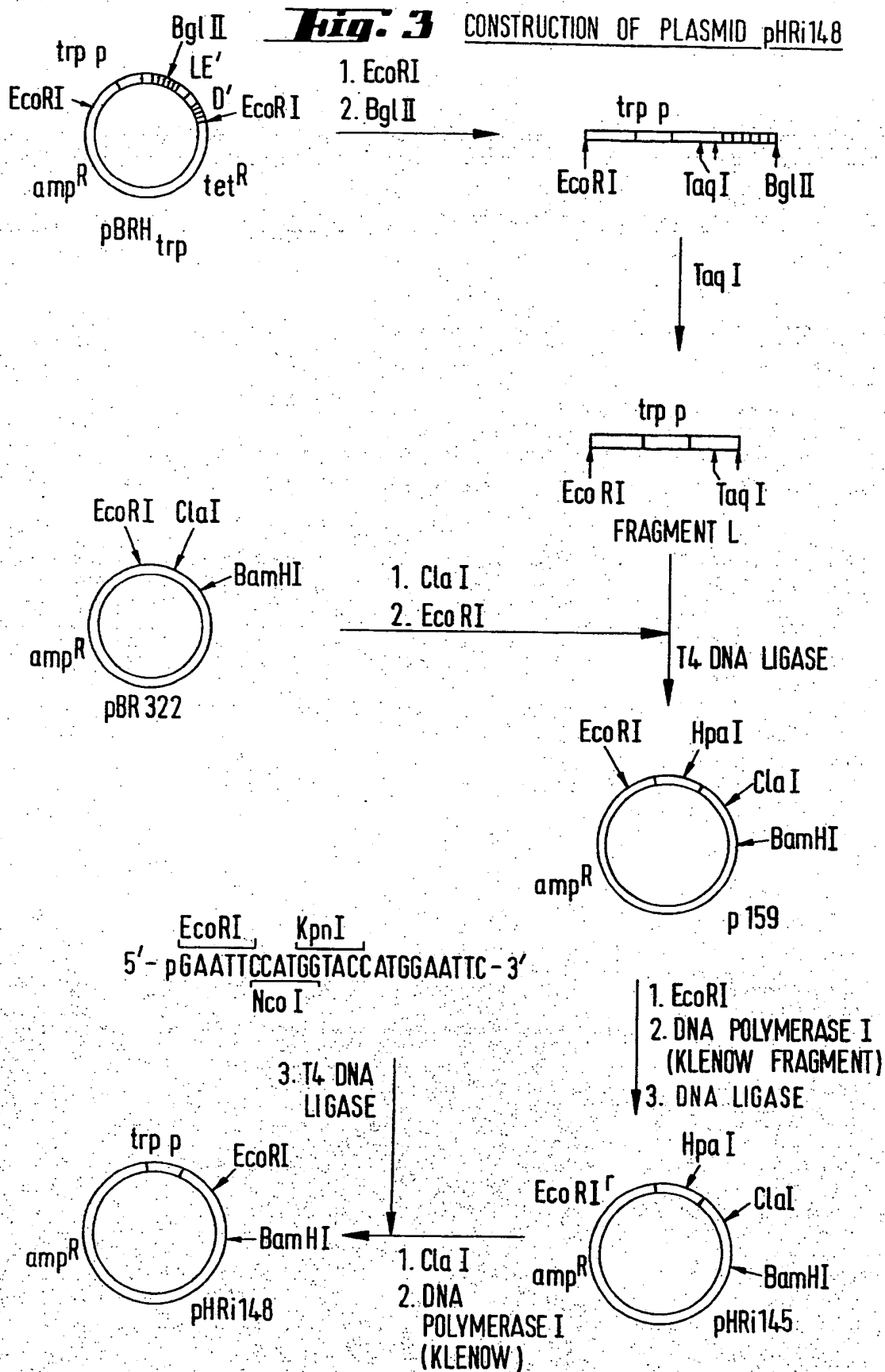


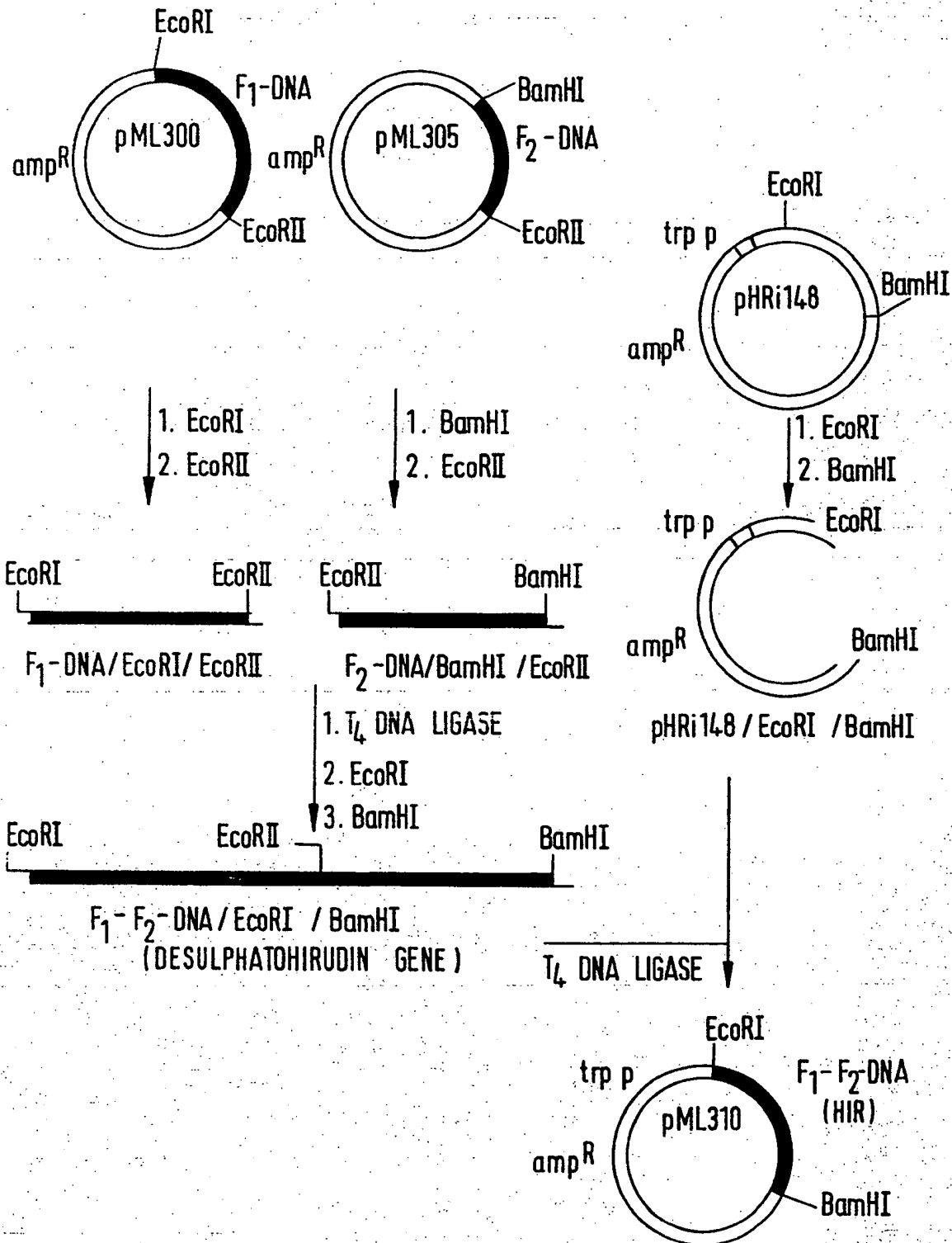
Fig. 4CONSTRUCTION OF EXPRESSION PLASMID pML310

Fig. 5 ISOLATION OF A DNA FRAGMENT CODING
FOR MATURE DESULPHATHIRUDIN

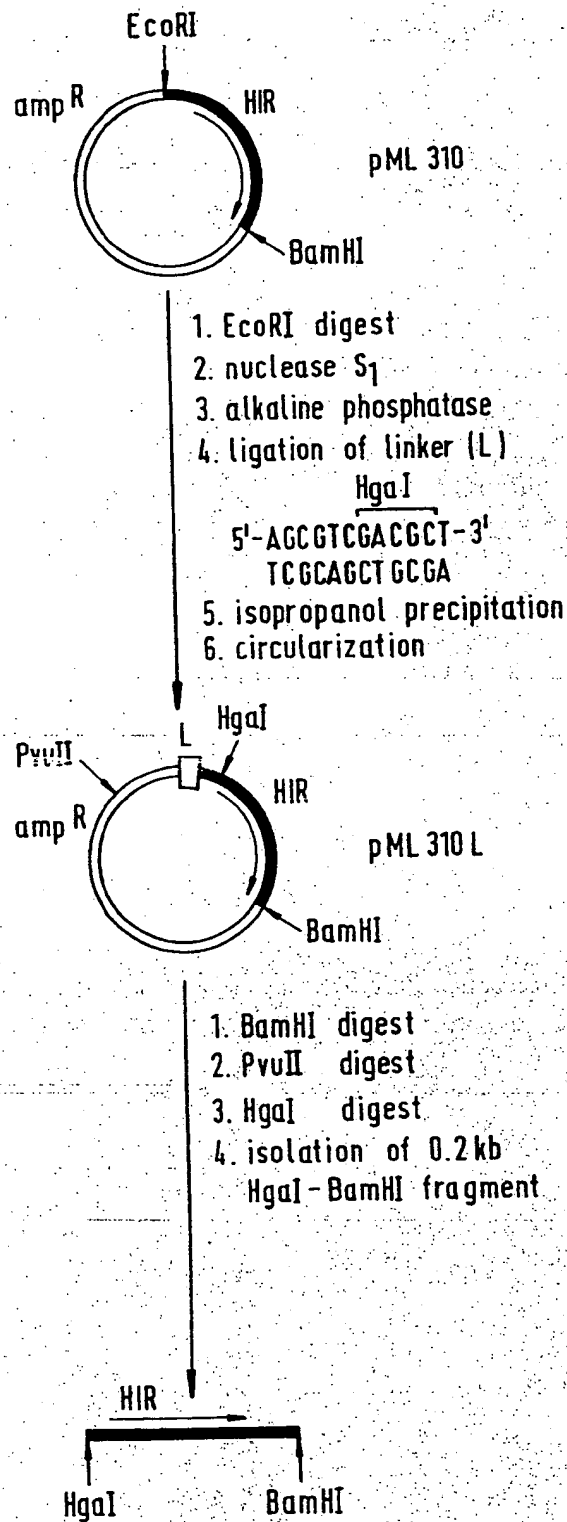
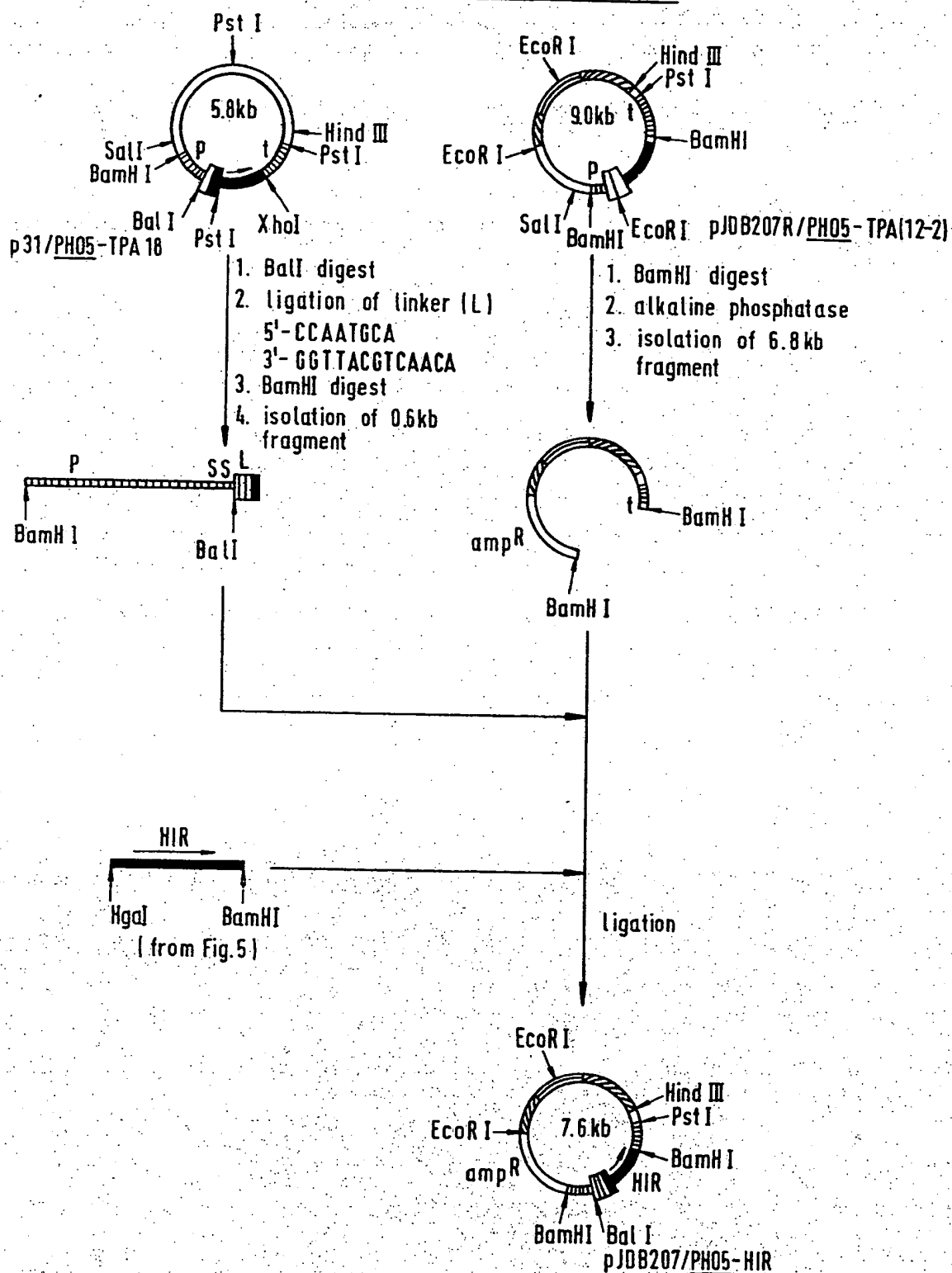
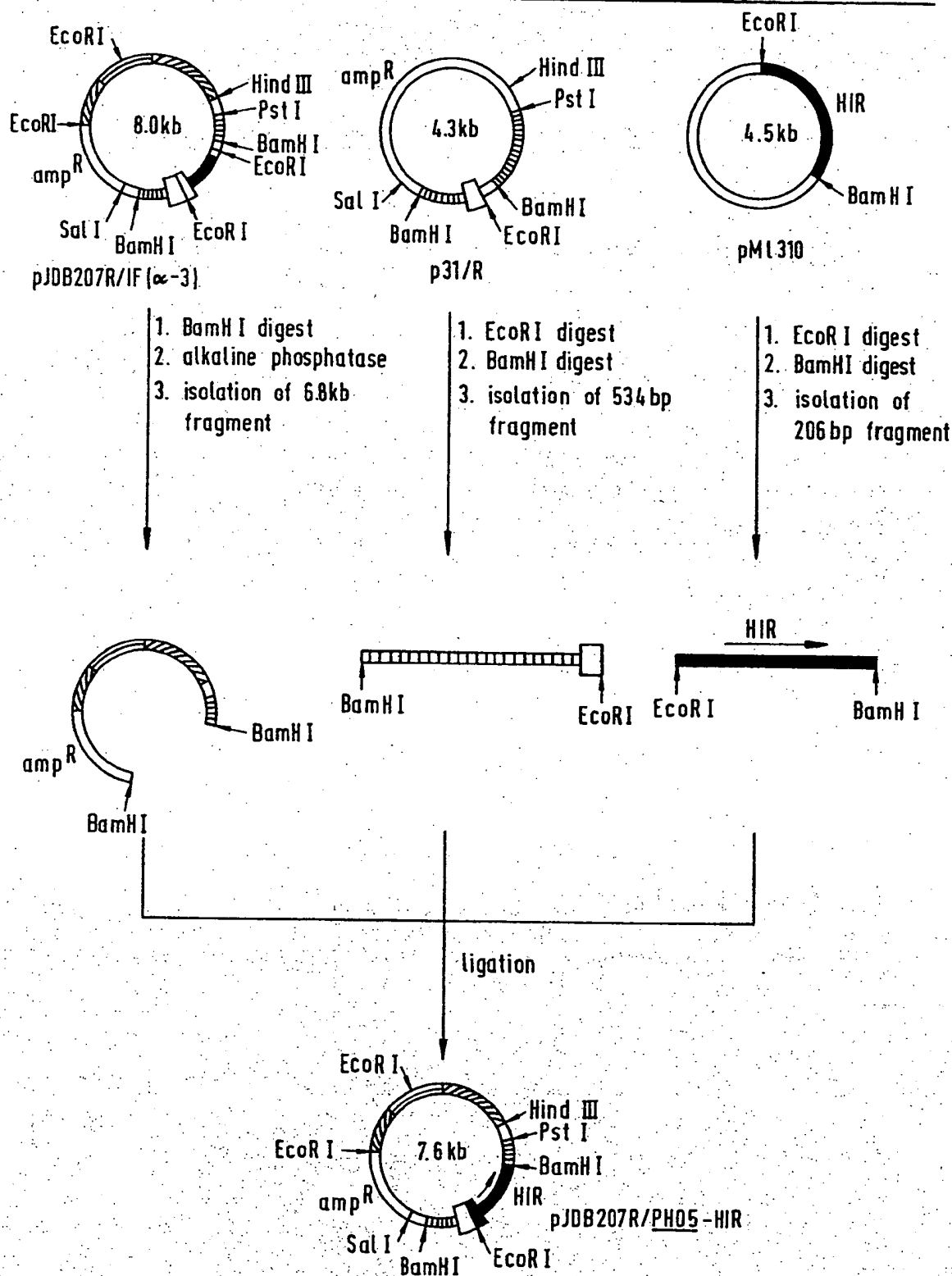


Fig. 6 CONSTRUCTION OF EXPRESSION PLASMID pJDB207/PH05-HIR
FOR SECRETION OF DESULPHATOHIRUDIN



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Fig. 7 CONSTRUCTION OF EXPRESSION PLASMID pJDB207R/PH05-HIR
FOR INTRACELLULAR EXPRESSION OF DESULPHATHIRUDIN



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Fig. 8 DNA SEQUENCE OF THE BamHI-SalI
RESTRICTION FRAGMENT OF PH05
INCLUDING THE PH05 PROMOTER
REGION

BamHI
↓
GG

-540 -530 - 520 -510 -500
 ATCCGAAAGT TGTATTCAAC AAGAATGCGC AAATATGTCA ACGTATTTGG

-490 -480 -470 -460 -450
 AAGTCATCTT ATGTGCGCTG CTTTAATGTT TTCTCATGTA AGCGGACGTC

-440 -430 -420 -410 -400
 GTCTATAAAC TTCAAACGAA GGTAAAAGGT TCATAGCGCT TTTTCTTTGT

-390 -380 -370 -360 -350
 CTGCACAAAG AAATATATAT TAAATTAGCA CGTTTTTCGCA TAGAACGCAA

-340 -330 -320 -310 -300
 CTGCACAATG CCAAAAAAAG TAAAAGTGAT TAAAAGAGTT AATTGAATAG

-290 -280 ClaI -270 -260 -250
 GCAATCTCTA AATGAATCGA TACAACCTTG GCACTCACAC GTGGGACTAG

-240 -230 -220 -210 -200
 CACAGACTAA ATTTATGATT CTGCTCCCTG TTTTCCAAGA GATCGCACAT

-190 -180 BstEII-170 -160 -150
 GCCAAATTAT CAAATTGGTC ACCTTACTTG GCAAGGCATA TACCCATTG

-140 -130 -120 -110 -100
 GGATAAGGGT AAACATCTTT GAATTGTCGA AATGAAACGT ATATAAGCGC

-90 -80 -70 -60 -50
 TGATGTTTTG CTAAGTCGAG GTTAGTATGG CTTTCATCTCT CATGAGAATA

-40 -30 -20 -10 -1 10
 AGAACAACAA CAAATAGAGC AAGCAAATTC GAGATTACCA ATGTTTAAAT

20 30 40 50 60

CTGTTGTTTA TTCAATTTTA GCCGCTTCTT TGGCCAATGC AGGTACCATT

70 80 SalI
 ↓

CCCTTAGGCA AACTAGCCGA TGTCGAC

Fig. 9 THE PROMOTER REGION OF THE GAPDH
GENE

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      TaqI  -670      -660      -650      -640
      TCGAGT TTATCATTAT CAATACTCGC CATTTCAAAG AATACGTAAA
-630      -620      -610      -600      -590
      TAATTAATAG TAGTGATTTT CCTAACTTTA TTTAGTCAAA AAATTAGCCT
-580      -570      -560      -550      -540
      TTTAATTCTG CTGTAACCCG TACATGCCAA AATAGGGGGC GGGTTACACA
-530      -520      -510      -500      -490
      GAATATATAA CACTGATGGT GCTTGGGTGA ACAGGTTTAT TCCTGGCATC
-480      -470      -460      -450      -440
      CACTAAATAT AATGGAGCCC GCTTTTAAAG CTGGCATCCA GAAAAAAAAA
-430      -420      -410      -400      -390
      GAATCCCAGC ACCAAAATAT TGTTTTCTTC ACCAACCATC AGTTCATAGG
-380      -370      -360      -350      -340
      TCCATTCTCT TAGCGCAACT ACAGAGAACA GGGCACAAAC AGGCAAAAAA
-330      -320      -310      -300      -290
      CGGGCACAAC CTCAATGGAG TGATGCAACC TGCCTGGAGT AAATGATGAC
-280      -270      -260      -250      -240
      ACAAGGCAAT TGACCCACGC ATGTATCTAT CTCATTTTCT TACACCTTCT
-230      -220      -210      -200      -190
      ATTACCTTCT GCTCTCTCTG ATTTGGAAAA AGCTGAAAAA AAAGGTTGAA
-180      -170      -160      -150      -140
      ACCAGTCCC TGAAATTATT CCCCTACTTG ACTAATAAGT ATATAAAGAC
-130      -120      -110      -100      -90
      GGTAGGTATT GATTGTAATT CTGTAAATCT ATTTCTTAAA CTCTTAAAT
-80      -70      -60      -50 DraI  -40
      TCTACTTTTA TAGTTAGTCT TTTTTTTAGT TTTAAAACAC CAAGAACTTA
-30 TaqI  -20      -10      -1
      GTTTCGAATA AACACACATA AATAAACAAA ATG

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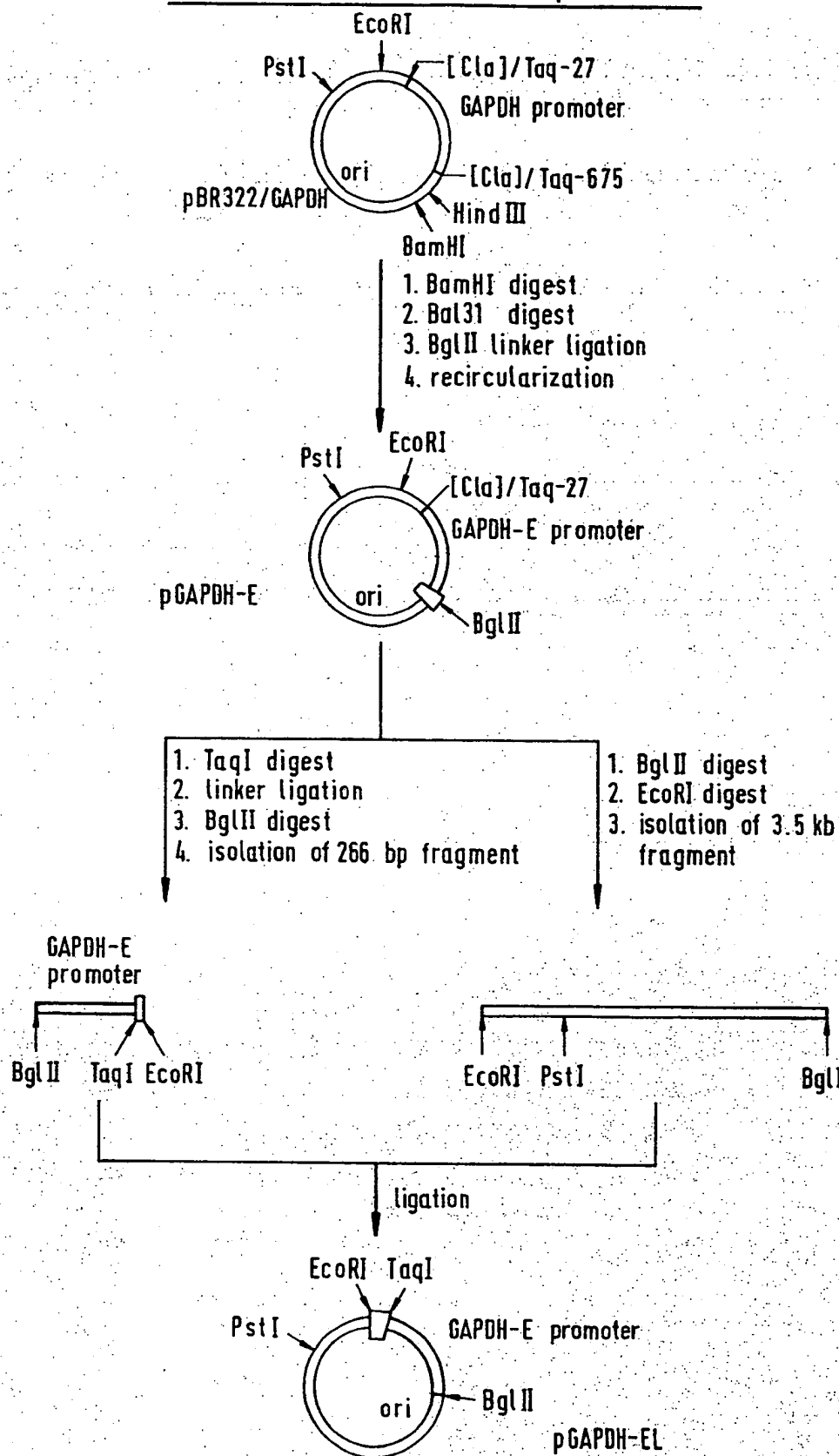
Fig. 10 CONSTRUCTION OF PLASMID pGAPDH-EL

Fig. 11 SEQUENCE OF BglII-EcoRI FRAGMENTS FROM
PLASMIDS pGAPDH-FL AND pGAPDH-EL,
RESPECTIVELY, COMPRISING PART OF THE
GAPDH PROMOTER

a. pGAPDH-FL

5'-GATC TGCTGAAAAA AAAGGTTGAA
ACCAGTTCCC TGAAATTATT CCCCTACTTG ACTAATAAGT ATATAAAGAC
GGTAGGTATT GATTGTAATT CTGTAAATCT ATTTCTTAAA CTTCTTAAAT
TCTACTTTTA TAGTTAGTCT TTTTTTTAGT TTAAAAACAC CAAGAACTTA
GTTTCGAATA AACACACATA AATAAAG-3'

b. pGAPDH-EL

5'-GATCTGCGC ATGTATCTAT CTCATTTTCT TACACCTTCT
ATTACCTTCT GCTCTCTCTG ATTTGGAAAA AGCTGAAAAA AAAGGTTGAA
ACCAGTTCCC TGAAATTATT CCCCTACTTG ACTAATAAGT ATATAAAGAC
GGTAGGTATT GATTGTAATT CTGTAAATCT ATTTCTTAAA CTTCTTAAAT
TCTACTTTTA TAGTTAGTCT TTTTTTTAGT TTAAAAACAC CAAGAACTTA
GTTTCGAATA AACACACATA AATAAAG-3'

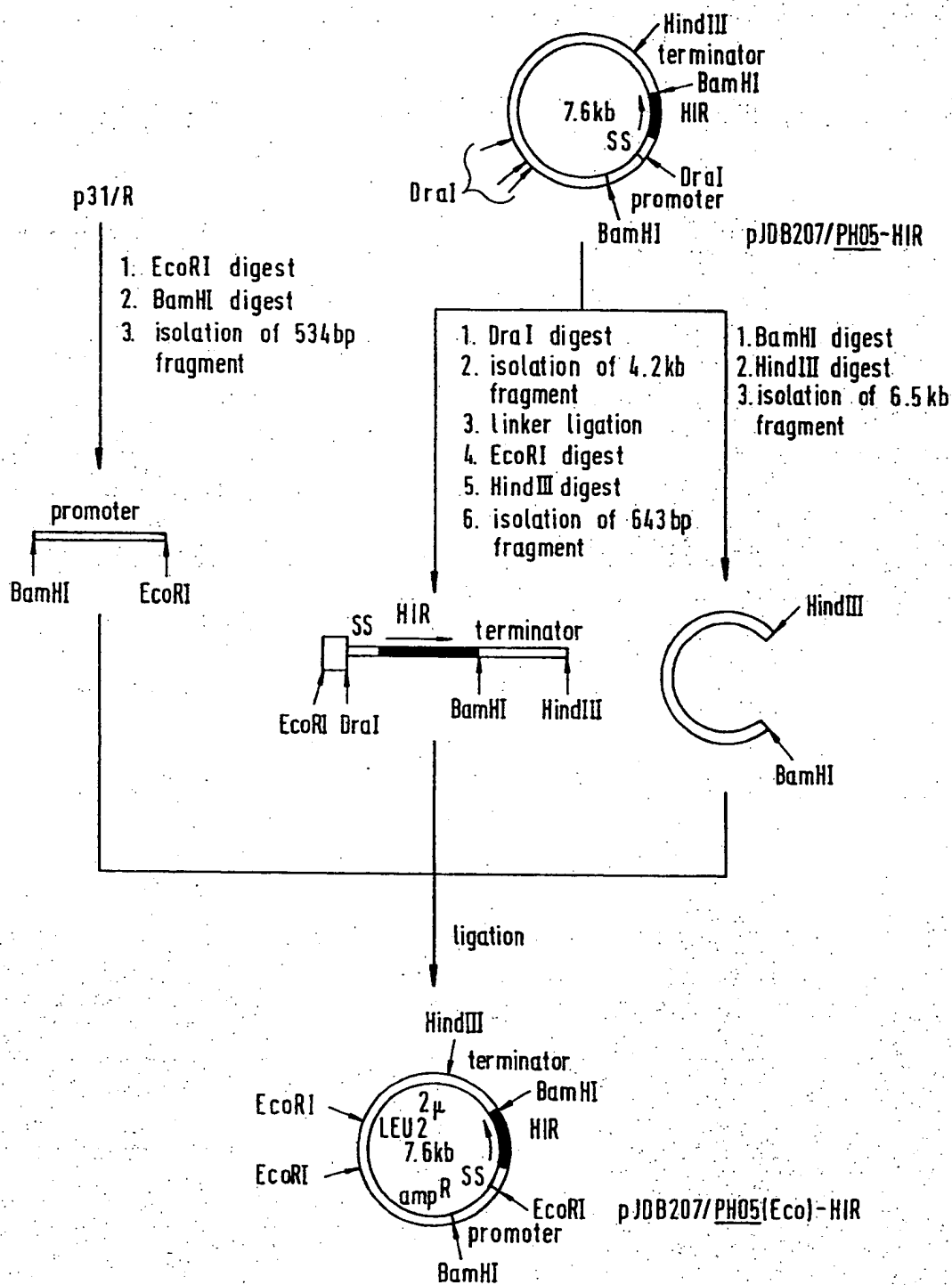
Fig. 12 CONSTRUCTION OF PLASMID pJDB207/PH05(Eco)-HIR

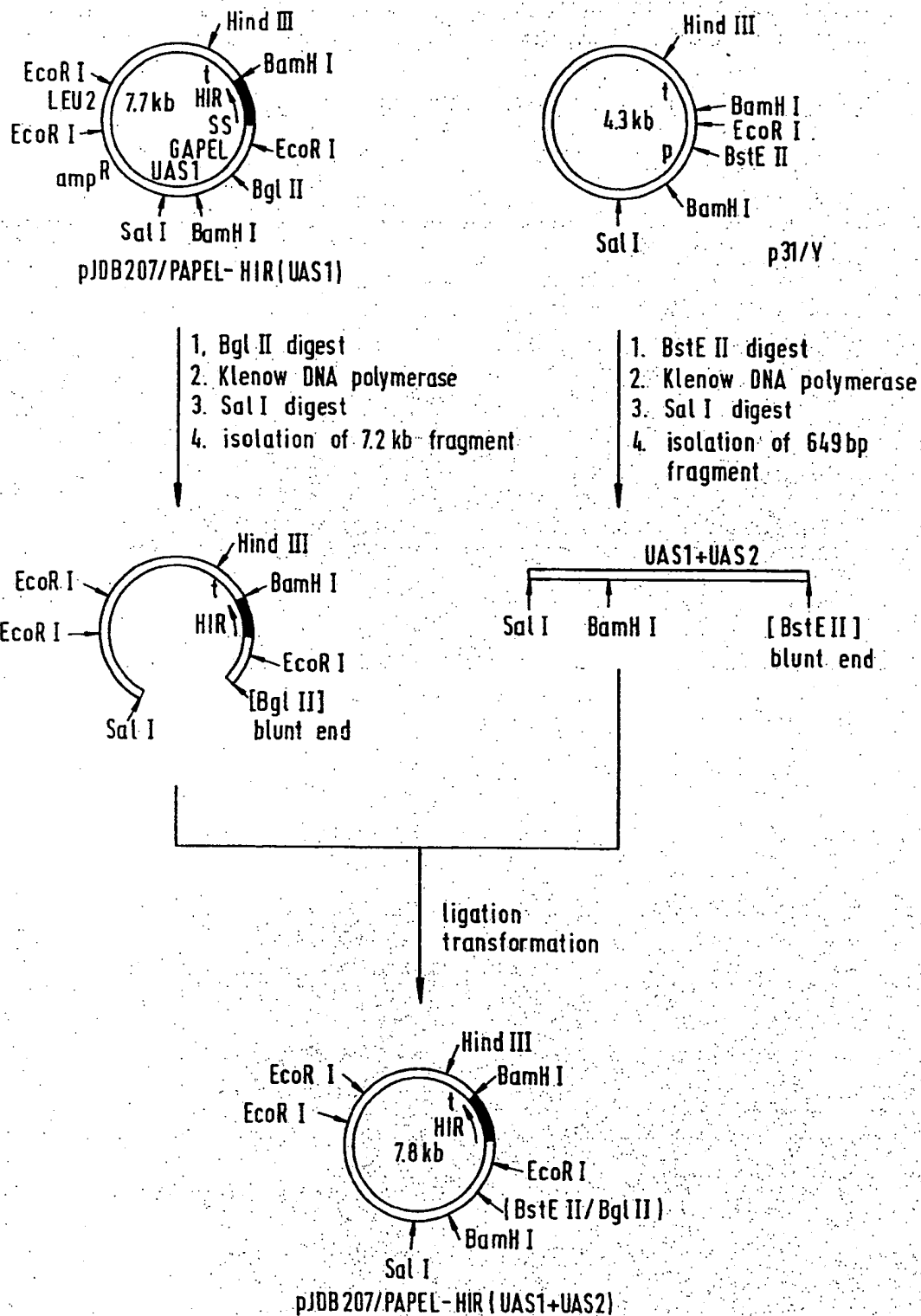
Fig. 13 CONSTRUCTION OF PLASMID pJDB207/PAPEL-HIR (UAS1+UAS2)

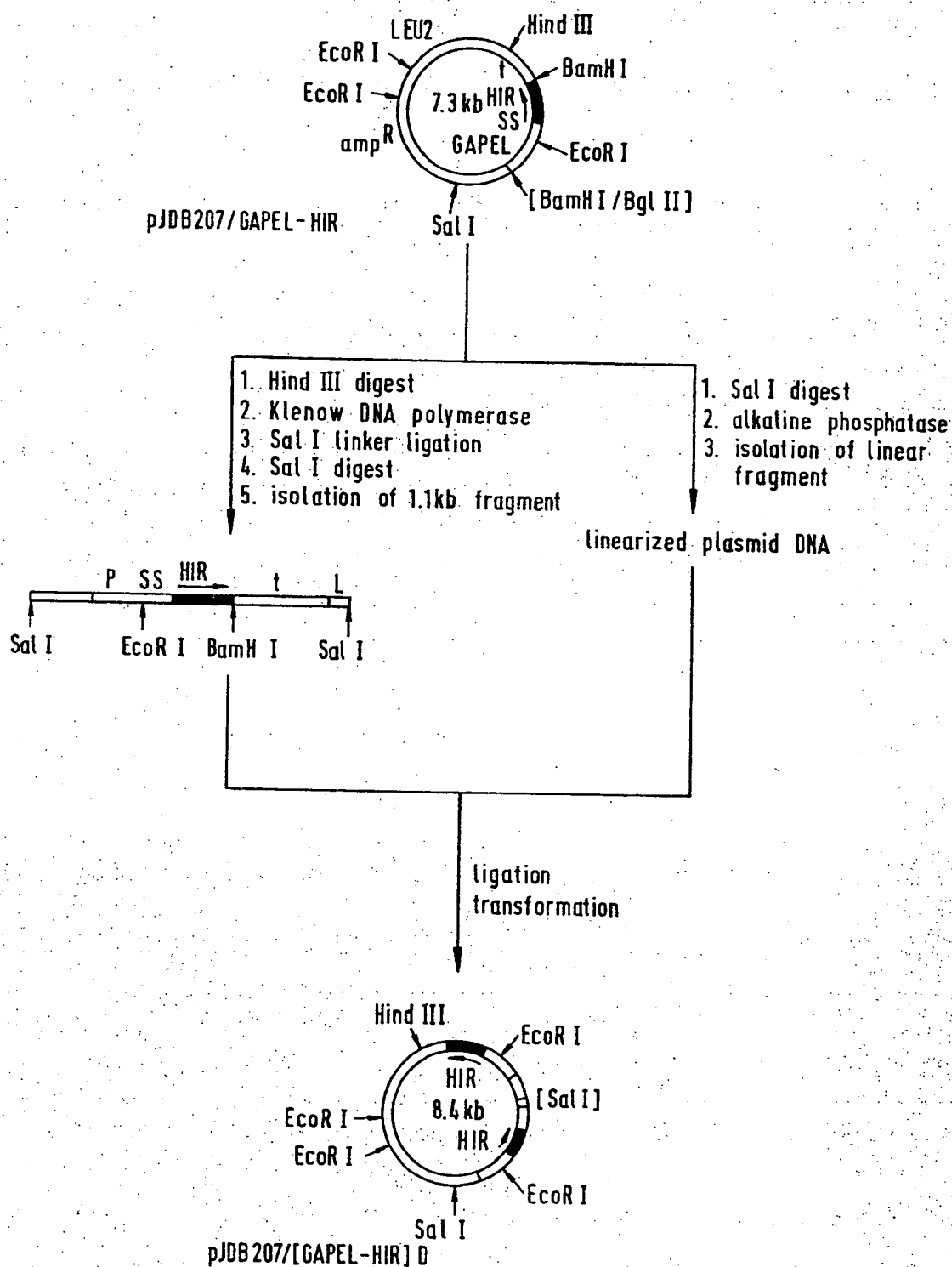
Fig. 14 CONSTRUCTION OF PLASMID pJDB207/[GAPEL-HIR]D

Fig. 15 CONSTRUCTION OF PLASMID pJDB 207/GAPFL-I-HIR